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Marfey's reagent for chiral amino acid analysis: A review

Review Article

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Summary. The present paper describes characteristics and application of Marfey's reagent (MR) including general protocols for synthesis of the reagent and diastereomers along with advantages, disadvantages and the required precautions. Applications, and comparison with other derivatizing agents, for the resolution of complex mixtures of DL-amino acids, amines and non-proteinogenic amino acids, peptides/amino acids from microorganisms, cysteine residues in peptides, and evaluation of racemizing characteristics have been discussed. Separation mechanisms of resolution of amino acid diastereomers and replacement of Ala–NH_2 by suitable chiral moieties providing structural analogs and different chiral variants and their application as a derivatizing agent to examine the efficiency, and reactivity of the reagent have been focussed. Use of MR for preparing CSPs for direct enantiomeric resolution has also been included.

Keywords: Marfey's reagent (1-Fluoro-2,4-dinitrophenyl-5-L-alanine amide, FDAA) – Pre-column derivatization – Enantiomeric resolution – Amino acids, proteinogenic, non-proteinogenic – HPLC – Chiral variants – Separation mechanisms

Abbreviations: AA, Amino acid(s); AAA, α-Alkyl amino acid(s); BGIT, 2,3,4,6-tetra-*O*-benzoyl-β-D-glucopyranosyl isothiocyanate; CDR, Chiral derivatizing reagent; CMPA, Chiral mobile phase additive; CSP, Chiral stationary phase; CZE, Capillary zone electrophoresis; Dab, 2,4-Diamino-*n*-butyric acid; DFDNB, 1,5-Difluoro-2,4-dinitrobenzene (Sanger's reagent); DAA-, 2,4-Dinitrophenyl-5-L-alanine amide-; DLA-, 2,4-Dinitrophenyl-5-L-leucine amide; DNFB, 2,4-Dinitrofluorobenzene; DNP-, 2,4-Dinitrophenyl-; DMSO, Dimethyl sulphoxide; DNPA-, 2,4-Dinitrophenyl-5-L-alanine amide (-amino acid); FAB, Fast atom bombardment; FDAA, Fluorodinitrophenyl-5-L-alanine amide; FDLA, Fluorodinitrophenyl-5-L-leucine amide; FDVA, Fluorodinitrophenyl-5-L-valine amide; FDNP, Fluorodinitrophenyl-; FDNPA, 1-Fluoro-2,4-dinitrophenyl-5-alanine; FDPEA, 1-fluoro-2,4-dinitrophenyl-5-(*R*,*S*)-phenylethylamine; GITC, 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate; ESIMS, Electro spray ionization mass spectrometry; MR, Marfey's reagent, (1-fluoro-2,4-dinitrophenyl-5-L-alanine amide); OPA, *ortho*-phthaldialdehyde; TEAP, Triethylammonium phosphate; TFA, Trifluoroacetic acid

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1 Introduction

The idea of stereochemistry and stereoselectivity goes back to Pasteur (1848), van't Hoff and Le Bell (1874) and since then separation of enantiomers has always been regarded as one of the most challenging problems for chemists/scientists working in the fields of chromatography, asymmetric synthesis, mechanistic studies, studies of structure-function relationship of proteins, pharmacology, medicine, extraterrestrial chemistry, life sciences etc.

About 20 genetically encoded amino acids are the building blocks of proteins that are the most important constituents of all living systems. Multicellular organisms usually have L-amino acids. Racemization of optically active amino acids in dilute acid or base or at neutral pH may take place even in the metabolically stable proteins of living mammals; as a consequence the protein structure-function relationship may be altered. The preparation of enantiomerically pure substituted analogs of amino acids is a challenging task and also requires accurate analytical method to determine enantiomeric excess during the course of asymmetric synthesis.

A multitude of synthetic peptides have been produced by resource and research laboratories for both basic research and drug discovery programs; this number would increase to myriads by inclusion of combinatorial libraries. Improved synthetic procedures and analytical technologies provide desired sequence and purity while little or no concern is generally given to stereoisomeric purity. It is often assumed that racemization may not occur or it need not be examined. Though, it is well known that the L and D form of

peptides can vary significantly with respect to stability and biological activity. Therefore, for example, if a significant portion of a synthesis is racemized and the racemized peptide is 1000 times more stable *in vivo*, the majority of the immune response may be directed to the racemized peptide and not to the desired peptide. Examining purity or racemization remains an analytical challenge. Thus, evaluation of the degree to which racemization occurs in peptides produced is required all the time. Although it is well known that in particular in bacteria peptidoglycan bonded as well as free D-amino acids are common it has not been recognized until recently that D-enantiomers do also occur in plants and animals including man.

Chromatography has progressed from the time of classical column in 1906 to the capillary electrochromatography (CEC) in 2000. HPLC has passed through many developmental phases, decreasing separation time due to reduction in particle size from >100 to $3\,\mu$m, improvements in pumping systems facilitating gradient elution, introduction of computer controlled equipment with an integrated data system, and microcolumn or narrowbore columns requiring small sample volumes and analyte weights etc. Separation of a variety of molecules, including diastereomers, on C8 or C18 RP columns, and direct enantiomeric resolution on bonded phase packings such as polysaccharides, cyclodextrins and macrocyclic antibiotics have been in practice during the last few decades. These CSPs eventually led to the decline of ligand – exchange chromatography. Although in this review we focus on liquid chromatographic methods based on the use of MR it is worth mentioning that gas chromatography proved to be one of the first methods resolving completely achiral derivatives (*e.g.*, volatile analytes by esterification) of DL-amino acids on chiral stationary phase which led to the development of Chirasil-Val as a GC CSP (Frank et al., 1977); possible thermal degradation of of the analytes and enantiomer selectivity bias were considered to be the main weaknesses.

2 Direct and indirect separation

There have been two basic approaches for the chromatographic resolution of enantiomers: a direct and an indirect method. The resolution of a pair of enantiomers by reacting them with an optically pure chiral reagent, *i.e.* the formation of diastereomers followed by their separation by chromatography in an achiral environment, is considered as an indirect approach and has been the most common means of achieving the resolution. The advantages include the commercial availabilty of a large number of chiral derivatizing reagents and a greater choice of chromatographic conditions. The enantiomer molecule and the chiral derivatizing reagent (CDR) must possess an easily derivatizable and compatible functional group. The reaction should be quick otherwise a variation in the formation rate of diastereomers may cause a kinetic resolution. Introduction of a chromophore enhances detection for HPLC resolution. Resolution via diastereomer formation is usually improved when bulky groups are attached to the chiral centre and when the chiral centres of both the reagent and the analyte are in close proximity in the resulting diastereomer (Stevenson and Williams, 1988). Various types of columns, mobile phases, and CDRs for the resolution of enantiomers of amino acids and their derivatives have been summarised (Bhushan and Joshi, 1993).

The direct approach requires no chemical derivatization prior to separation process. Resolution is possible through reversible diastereomeric association between the chromatographic chiral environment and the solute enantiomers. The enantiomers may interact during the course of chromatographic process with a chiral stationary phase (CSP) or a chiral selector added to the mobile phase (CMPA) or a chiral selector mixed with/immobilised (especially in TLC) on the stationary phase (Bhushan and Martens, 1997, 2003). Some important binding types present in enantioselective sorption process include, coordination to transition metals (ligand exchange), charge transfer interaction, ion exchange, and inclusion phenomena (host-guest complex). Use of CSP in GC required achiral derivatization (and protection of side chain functionality) to produce volatile analytes, *e.g.* esterification of amino acids; possible thermal degradation, racemization and enantiomer selectivity bias have been the main weaknesses. Direct HPLC resolution of enantiomers of amino acids using various CSPs, and CMPAs, has been summarised by Bhushan and Joshi (1993). Direct methods have certain critical disadvantages. Protein stationary phases are not durable over time and pH and also have low sample capacity. Besides, the correct elution order is difficult to be predicted because of the complexity of interactions with the protein (Pirkle and Pochapsky, 1989). Stationary phases with crown ethers and cyclodextrins, involving host-guest type complexation, often result in poor band shape and have slow kinetics on a chromatographic time scale.

The separation of diastereomeric pair via the indirect technique is sometimes simpler to perform and often has better resolution than with a direct method because chromatographic conditions are much easily optimized. MR has such attributes.

Keeping in view the scope of this article to resolution of enantiomers of amino acids by Marfey's reagent, references to enantiomeric resolution of several other classes of compounds, including amino acids, by different chromatographic techniques involving both direct and indirect approaches, are not being included. However, the books and articles covering the whole subject of chiral resolutions may also be referred to. Since B'Hymer et al. (2003) recently discussed uses of MR mainly to resolution of amino acids, the focus of the present paper is on mechanistic aspects of resolution of amino acid diastereomers and on the application of MR as a derivatizing agent in the form of different chiral variants. Besides, advantages, disadvantages along with required precautions, and application to resolution of complex mixtures of DL-amino acids, amines and non-proteinogenic amino acids, peptides/amino acids from micro-organisms, and evaluation of racemizing characteristics have been discussed.

3 Marfey's reagent (MR)

There is little doubt that of the various methods available for the indirect HPLC resolution of enantiomers of amino acids use of MR has been most successful. The reagent meets satisfactorily the characteristic features, as enumerated above, as a derivatizing agent in different situations. Marfey's method has been widely used for structural characterization of peptides, confirmation of racemization in peptide synthesis, and detection of small quantities of D-amino acids. Many other reagents used for pre-derivatization of free amino acids suffer inherent problems, *e.g.*, inability to react with all proteinogenic amino acids, unstable derivatives, poor detectability of certain amino acid derivatives, or lack of quantitative yield of the reaction.

TLC has also been successful in resolving MR derivatives of 22 DL-amino acids (DL-ethionine, DL-citrulline and 20 proteinogenic amino acids) on C18 silica layers though it was not possible to separate all 22 amino acid derivatives (*i.e.* 44 individual compounds) in a single conventional run due to a large number of overlapping R_f values (Ruterbories and Nurok, 1987). A few other RP-TLC separations of amino acids using MR include, glutamate and aspartate effectively with solvent consisting of 25% acetonitrile in TEP buffer (50 mM, pH 5.5), L- and D-serine with 30% acetonitrile as solvent, enantiomers of Thr, Pro, and Ala with 35% acetonitrile, and those of Met, Val, Phe and Leu with 40% of acetonitrile as solvent (Nagata et al., 2001).

3.1 Synthesis and derivatization

Marfey was the first to synthesise 1-fluoro-2,4-dinitrophenyl-5-L-alanineamide (FDAA) in high yield (76%) by the reaction of 1,5-difluoro-2,4-dinitrobenzene (DFDNB) and L-Ala–NH_2. It is popularly known as Marfey's Reagent (MR). Substitution of one of the two fluorine atoms in 1,5-difluoro-2,4-dinitrobenzene by L-alanine amide yields the said reagent. It reacts stoichiometrically, without racemization, within 1 hr under alkaline conditions at 40°C with the α-amino group of L- and D-amino acids yielding diastereomers (Fig. 1a). These diastereomers can be separated and estimated (in nanomole range) by HPLC due to large difference in their capacity factors. The amide was chosen because it is quite stable, neutral and apparently is not easily racemized.

The kinetics of the reaction of MR with amino acids was identical to the reaction kinetics of amino acids with 2,4-DNFB and the yield of the reaction was more than 99% as determined with radio actively labeled amino acids.

3.2 General protocols for synthesis

The cost of synthesis in terms of the cost of only reactants may be 50 times less than that of the commercial reagent. The general approach to synthesise the reagent (if not obtained commercially) and derivatization is that reported by Marfey (1984). However, certain modifications have been reported from time to time. Some of these are described below as a ready reference for the readers.

(a) Synthesis of FDNP–L-Ala–NH_2 (FDAA), (Marfey, 1984)

A sample of L-Ala–$NH_2 \cdot HCl$ (472 mg, 3.81 mmol) is dissolved in NaOH (1 M, 3.9 ml) and immediately acetone is added (60 ml). After adding anhydrous $MgSO_4$ (about 10 g) the contents are stirred at room temperature for about 3 hr. $MgSO_4$ is removed by filtration and washed twice with a little acetone. DFDNB (668 mg, 3.27 mmol) is dissolved in acetone (15 ml) and to it the solution of L-Ala–NH_2 (in acetone) is added dropwise under magnetic stirring. The contents are then stirred for 30 min. Equal volume of water is added when golden yellow scales are formed. These are filtered, washed first with little 2:1 water–acetone mixture, then with water and finally dried in air in the dark. The yield is 0.5 g (56%, mol wt 272), m.p., 224–226°C. Another crop of crystals is obtained from the mother liquor upon removal of more acetone under vacuum. TLC in ethyl acetate as solvent, and HPLC

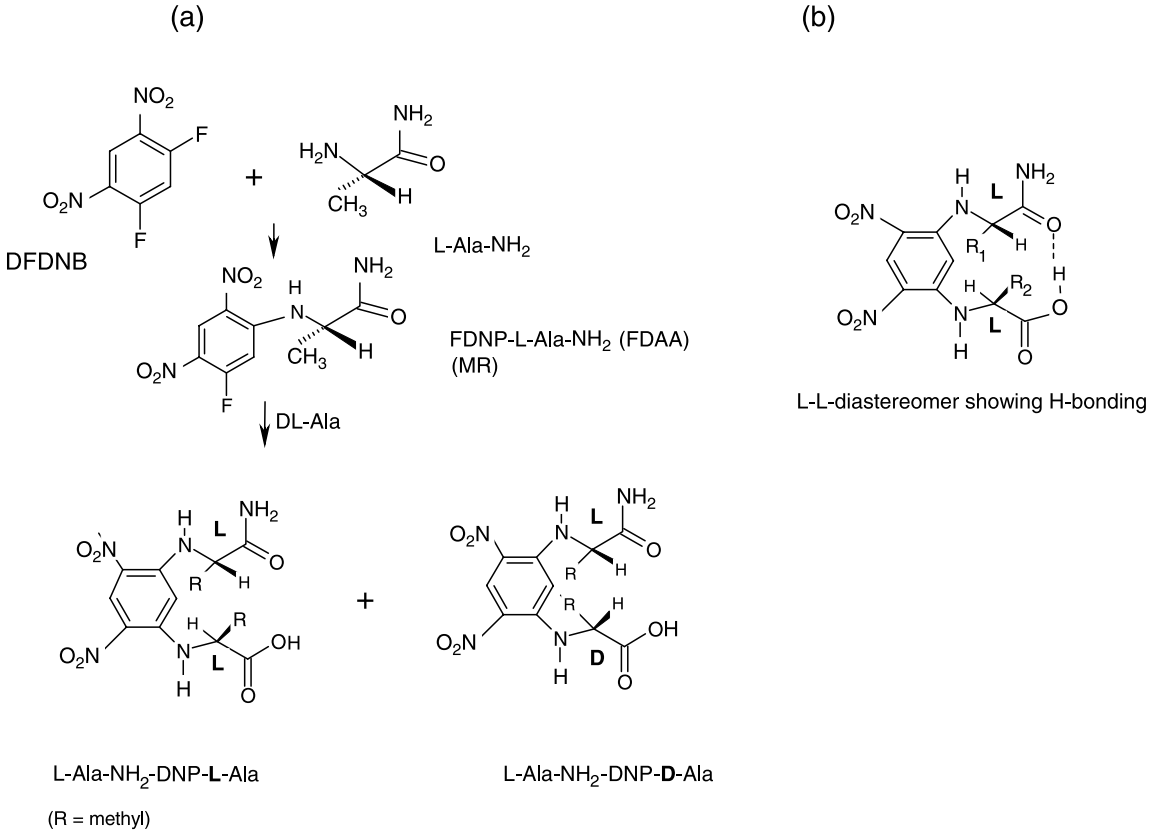

Fig. 1. (**a**) Reaction sequence for the synthesis of FDAA and formation of L-L- and L-D-diastereomers. (**b**) Structure of L-L-diastereomer showing H-bonding (Brückner and Keller-Hoehl, 1990)

using 50 mM TEAP/acetonitrile (pH 3.0, from 10% to 50% acetonitrile during one hour, flow rate 2 ml/min, at 340 nm) confirm the purity. The UV in 25 mM TEAP, pH 3.0 and 50% acetonitrile shows a maxima at 264 nm, 338 nm and shoulder at 380 nm.

Using modifications of the above protocol, Fujii et al. (1997a) prepared FDNP–L-Val–NH_2 (FDNPVA), FDNP–L-Phe–NH_2, FDNP–L-Ile–NH_2, FDNP–L-Leu–NH_2 (FDNPLA), and FDNP–D-Ala–NH_2, while Brückner and Keller-Hoehl (1990) prepared FDNP–L-Ala–NH_2, FDNP–L-Val–NH_2, FDNP–L-Phe–NH_2, and FDNP–L-Pro–NH_2 with minor changes in reaction conditions, *e.g.*, heating the reaction mixture at 40 to 50°C for 1 to 2 hr in some cases.

(b) Synthesis of L- and D-diastereomers of amino acids

Derivatization of an amino acid with FDAA produces a diastereomer referred to as 2,4-dinitrophenyl-5-L-alanin-amide amino acid or simply DNPA-amino acid. Aqueous solutions (50 mM) of amino acids (D- and L-isomers) are used as starting materials for synthesis. Solution of each of the amino acid (50 μl, 2.5 micromoles) is placed in separate 2 ml plastic tubes. To each is added 100 μL of 1% acetone solution of FDAA (1 mg, 3.6 micromoles), the molar ratio of FDAA to amino acid 1.4:1, followed by $NaHCO_3$ (1 M, 20 μL, 20 micromoles). The contents are mixed and heated over a hot plate at 30–40°C for 1 hr with frequent mixing. After cooling to room temperature, HCl (2 M, 10 μl, 20 micromoles) is added to each reaction mixture. After mixing, the contents are dried in a vacuum desicator over NaOH pellets. Each residue is then dissolved in DMSO (0.5 ml). A 1:1 dilution of these is made (2.5 mM) and 5 μl sample of each is pooled and injected for HPLC.

It has been recognized that addition of DMSO or the use of mixtures of triethylamine and DMSO drastically increased the reaction rate of derivatization and are recommended to overcome the low reactivities in the case of sterically hindered reagents; the gradient elution with mixtures of sodium acetate buffer of pH 4.0 and methanol or *aq* TFA and acetonitrile are also suitable (Brückner and Keller-Hoehl, 1990).

Application of original protocol of Marfey (1984) was unsuccessful for the resolution of enantiomers of ring- and α-methyl substituted phenylalanines and phenylalanine

amides (Péter et al., 2000). In the modified procedure of derivatization, the analyte (1 mg) was dissolved in water (1 ml). An aliquot (100 μl) was mixed with a solution of FDAA in acetone (100 μl, 1.6%, w/v, molar ratio of FDAA to analyte about 15:1). Higher reactant concentration and longer reaction times were also used. The reaction was allowed to stand overnight at 40°C or for 6 hr at 50°C. The reaction was found to remain slow. It was stopped by addition of 2 *M* HCl and diluted twofold directly with the mobile phase. The application of a higher temperature and a longer reaction time may promote racemization of enantiomers which have hydrogen in α-position. Derivatization of peptides (*e.g.* oxytocin) with free α-amino group requires longer reaction time than that of amino acids (Szabó et al., 2001); instead of 90 min the reaction time for peptides is 12–24 hr and five times excess of reagent is necessary. The retention time of peptides increases significantly depending on the size of the peptide fragment.

3.3 Elution and detection

With the five amino acids studied by Marfey, elution was done with linear gradient of acetonitrile in 50 mM TEAP buffer, pH 3.0, from 10% to 50% acetonitrile during 1 hr, flow rate 2 ml/min, at 340 nm. L-diastereomers were eluted from the RP column before D-diastereomers and all the ten diastereomers had a very similar absorption spectra characterized by a λ_{max} at 340 nm. The λ_{max} values are slightly different for diastereomers of different amino acids and vary slightly with the nature of solvent used. The spectra are stable if the solutions are kept in dark, otherwise, a gradual change occurs as a result of a photochemical decomposition of the absorbing chromophore. The hydrolysed reagent appears as sharp peak and is separated from all the diastereomers. Detection at 340 nm make Marfey-derivatives insensitive to most buffer systems and most mobile phase impurities. The only detection interference is the excess MR itself. The chromatographic conditions can be adjusted to avoid co-elution with any of the desired analyte peaks.

The applicability and limitations of Marfey's method were extensively examined by Harada et al. (1995). Neutral, hydroxy, basic and *N*-methyl amino acids were examined. The neutral amino acids showed good resolution between the L- and D-diastereomers, while the separation for hydroxy and acidic amino acid derivatives was very poor. Basic amino acids gave three derivatives (the mono-α, mono-ω, and di-derivatives) and indicated that α-amino group is essential for the resolution of both diastereomers. The resolution power of *N*-methylamino acid FDAA derivatives decreased in comparison to that of their parent amino acids. For standard α-amino acids, except for a few basic amino acids, the Marfey's method proved to have a wide applicability.

3.4 Advantages

MR provides a very simple and effective analytical method. Hydrolysis of peptides or proteins (may be microwave assisted) followed by derivatization of the resulting amino acids with the chiral Marfey's reagent adds a highly absorbing chromophore that converts the amino acids into UV active diastereomers. This allows separation of D- and L-amino acids as diastereomers in the nanomole range on a nonchiral column with the inherent rapidity of determination in HPLC. In addition, these derivatives of amino acids can be detected in both, simple UV as well as more selective mass spectrometric devices.

The method is considered to have an advantage over the method of Manning and Moore (1968) in that it does not produce oligomeric products that can be formed when an L-amino acid *N*-carboxy anhydride (NCA) reacts with a mixture of L- and D-amino acids. Major advantages of Marfey's reagent over other pre-column derivatizations include (i) possibility to carry out chromatography on any multipurpose HPLC instrument without column heating, (ii) simultaneous detection of proline in a single chromatographic run, and (iii) stable amino acid derivatives.

The Marfey's-derivatives of D- and L-amino acids can be identified by co-injection of standard derivatized D- and L-amino acids; the reciprocity principle of chromatography makes it possible, using both enantioisomeric reagents, to determine the opposite stereoisomer's retention time without measuring for the corresponding authentic sample.

The other advantage of MR is the possibility to increase its hydrophobicity (thus increasing α and R_s of the derivatives), by replacing the chiral selector Ala–NH_2 of the reagent with other amino acids such as Leu–NH_2, or Val–NH_2 (Harada et al., 1993; Brückner and Keller-Hoehl, 1990; Brückner and Gah, 1991). This method can simultaneously identify amino acids with the correct absolute configuration under gradient elution conditions and is highly sensitive. Since D- and L-amino acids are separated by HPLC as diastereomers of MR and the L-amino acid derivative is usually eluted before the corresponding D-isomer the method is useful to determine absolute configuration.

These characteristics make the method quite flexible for resolution and quantitation of such optical isomers.

3.5 Disadvantages and precaution

Amino acids like tyrosine and histidine or containing two amino groups such as ornithine and lysine can form both mono- and di-substituted Marfey's derivatives thus doubling the number of peaks in a chromatogram for these amino acids. Using MR in slight excess mono-substituted derivative can be minimised. Reaction of hydroxyl group of tyrosine with MR produces both mono- and di-substituted derivatives (Brückner and Gah, 1991); increasing the strength of base during derivatization reaction increases the yield of disubstituted tyrosine and minimizes the appearance of the peak for mono-substituted derivative.

Enantiomeric purity of the reagent is very important when the reagent is produced synthetically; enantiomerically pure L-alanine amide must be used and no racemization must have occurred during the synthesis. A small quantity of D-isomer of MR can cause a false detection of the other enantiomer in the chromatographic analysis of the diastereomer. Purity of the final reagent is required to be determined by a direct chromatographic procedure. Determination of absolute configuration is difficult for a peptide containing unusual amino acids without having their standard samples.

4 Chiral variants of MR

The MR can safely be considered as a chiral variant of the Sanger's reagent (2,4-DNFB). The Sanger's reagent (1945) was unique because it provided corresponding DNP derivatives of amino acids that were identifiable by chromatography to establish amino acid sequence in peptides. But there was no focus at that time towards resolution of enantiomers, may be because that was not required for sequence determination and more importantly there was no concern about the enantiomeric resolution. Nevertheless, the DNP-amino acids are chiral molecules except for glycine.

The MR (FDNP–Ala–NH_2 or FDAA) takes advantage of the remaining reactive aromatic fluorine, that undergoes nucleophilic substitiution with the free amino group on L- and D-amino acids (in the mixture), peptide or target molecule, and that of the stereogenic centre in its alanine group (the L-form) to create diastereomers. It thus provides a structural feature to replace L-Ala–NH_2 by suitable chiral moieties such as those of other amino acids etc and have the analogs to examine efficieny and reactivity of the reagent.

Three chiral variants of MR were prepared by the reaction of DFDNB with Val–NH_2, Phe–NH_2 and Pro–NH_2. These FDNP-reagents made possible the resolution of 19 pairs of DL-proteinogenic AAs, containing neutral, acidic, basic or aromatic side chains, by HPLC as diastereomers and differences in retention times Δt_R of these diastereomers were compared with those obtained by derivatization of the same set of 19 DL-AAs with MR (Brückner and Keller-Hoehl, 1990). However, the FDNP–Val–NH_2 gave the largest Δt_R values.

Further variants in the form of substituted FDNP–Val amide, FDNP–Val esters, FDNP-amines and FDNP-dipeptide amide were prepared to determine the structural parameters responsible for larger Δt_R values of AA diastereomers and to optimise HPLC separation of diastereomers formed by reaction of these FDNP reagents with certain AAs (Brückner and Gah, 1991). The major variants prepared and studied were (a) FDNP–Val–CONH***R*** with the group ***R*** as, –H, –*t*-Bu, $-CH(CH_3)C_6H_5$, $-C_6H_5$, and –*p*-nitrophenyl, (b) FDNP–Val–CO***OR*** with the group ***OR*** as, –OH, $-OCH_3$, and –O–*t*-Bu, (c) FDNP–Ala–Ala–NH_2, and (d) FDNP–PEA and FDNP–Valol, (–Val or –Ala bonded to FDNP through α–NH–). The amino acids analysed were DL-Val, –Glu, –Ser, –Lys (mono), and –Lys (di). FDNP–Val–NH_2 has been reported to provide retention times varying between 4 and 20 min in comparison to FDAA for determination of configuration and stereochemical purity of cysteine residues in peptides (Szabó et al., 2001).

Based on considerations of structural analogy and reactivity Brückner and Strecker (1992) and Brückner and Wachsmann (2003) carried out nucleophilic substitution of the three halogens (chlorine or fluorine) of trihalo-*s*-triazines (2,4,6-trihalo-1,3,5-triazines), cyanuric chloride or cyanuric fluoride respectively, by (i) reaction with either methanol, 2-naphthol, 1-methoxy naphthalene, or 4-aminoazobenzene, providing uv absorbing chromogenic dihalo-*s*-triazines, (ii) reaction with L-Alanine amide yielding chiral monohalo-*s*-triazines, and (iii) by reaction (of the monohalo-*s*-triazine) with selected D- or L-amino acids to form diastereomeric derivatives, which were separated by RP (C18) HPLC using mixtures of water, acetonitrile, and TFA as eluents. Thus, the dinitrofluoro moiety of, *e.g.*, FDAA, was replaced by a *s*-triazine moiety having a suitable chromophore. These chiral monohalo-*s*-triazines were found capable of resolving certain DL-amino acids. The resolution was lower in some cases in comparison with those obtained by reaction of the same

DL-amino acids with other chiral variants, described above (Brückner and Keller-Hoehl, 1990; Brückner and Gah, 1991). Analysis of results suggested that the nitro groups in the dinitro fluoro moiety of (say, FDAA) was contributing to better resolution while the increasing bulkiness of the substituent R in the monohalo-*s*-triazine lead to decrease in resolution. Nevertheless, these variants provided a general approach for the design and construction of tailor made reagents suitable for precolumn derivatization and liquid chromatographic separation of resulting diastereomers of the amino acid enantiomers. The approach was extended to other CDRs (Brückner and Leitenberger, 1996).

Marfey's method has also been combined with mass spectrometry; FDAA was replaced by L-FDLA (1-fluoro-2,4-dinitrophenyl-5-L-leucinamide) and it was observed that it provided enhanced sensitivity, hydrophobicity, and thermal stability, in comparison to L-FDAA. Electrospray ionization and frit-fast atom bombardment (Frit-FAB) were applied as the interface (Harada et al., 1995; Fujii et al., 1997b). It also required to change the original mobile phase of acetonitrile-phosphate buffer to acetonitrile-TFA as the former was not volatile. Derivatized amino acids with FDLA showed almost the same retention behaviour as that with FDAA.

It was interesting to observe (Harada et al., 2001) that *bis*-DLA as well as *bis*-DAA derivatives of Orn showed an opposite elution order (D- followed by L-isomer) while the L-isomers of the *bis*-DAA derivatives of Dab (2,4-diamino-*n*-butyric acid), and Lys eluted prior to the corresponding D-isomer in HPLC. NMR and UV spectral analysis did not provide any significant structural information and efforts to obtain suitable crystalline forms for X-ray crystallography were also not successful. Therefore, the use of DPEA (2,4-dinitrophenyl-5-phenylethylamine) derivative is recommended for analysis of Orn instead of DLA derivative in spite of low resolution power.

Harada et al. (1998) prepared a chiral anisotropic reagent, 1-fluoro-2,4-dinitrophenyl-5-(*R*,*S*)-phenylethyl amine [(*R*,*S*)-FDPEA], for determination of absolute configuration of α-carbon of primary amino compounds. The reagent has advantages in terms of reactivity, reliability and effectiveness and has successfully been applied to a peptide (microgenin) produced by cyanobacterium. The reagent was prepared by Marfey's method (1984), while the derivatives were prepared by treatment of primary amino compounds with FDPEA under slightly basic conditions followed by TLC preparative separation. The amino compounds analysed include L-isoleucinol, D- and L-phenylalaninols.

5 Separation mechanisms

The separation of FDAA derivatives of amino acids has been presented in literature based on their different conformations. Marfey (1984), and Brückner and Keller-Hoehl (1990) and Brückner and Gah (1991) discussed independently that the resolution of L- and D-derivatives is essentially due to intramolecular H-bonding. Marfey's conclusions were based on the structural features and elution behaviour of five amino acid derivatives while those of Brückner et al. were based on structural features correlated with separation behavior of a large number of derivatives including several chiral variants (as mentioned above) and construction of molecular models. These are briefly described below.

Marfey (1984) attributed the reason for the L-diastereomer eluting before the D-isomer to a stronger intramolecular H-bonding in D- than in L-isomer. He suggested that the carboxy group can H-bond either to an *ortho*-situated nitro group producing a nine membered ring or, more likely, to the carbonyl oxygen of the *meta*-situated L-Ala–NH_2 forming a 12-membered ring. Stronger H-bonding in a D-diastereomer would produce a more hydrophobic molecule which would be expected to interact more strongly with the RP column and thus have a stronger retention time than an L-diastereomer. Nature of the amino acid side chain is also responsible for the differences in elution times of the diastereomers; the ionizable side chains of Asp and Glu decrease the separation while neutral and hydrophobic side chains increase it.

The construction of (space filling Corey-Pauling-Koltun) molecular models of the L–L and D-L diastereomers (the first letter refers to the configuration of AA to be analysed and the second to that of the reagent) showed (Brückner and Keller-Hoehl, 1990; Brückner and Gah, 1991) that

(i) in the L–L, the carboxyl group of the analyte is located extremely close to the carboxamide of the reagent, thus facilitating the formation of an intramolecular H-bridge (Fig. 1b). Formation of a H-bridge between the carboxyl and the carboxamide group is not possible to such an extent in the case of D-L diastereomer. The non H-bonded free carboxy group of the D-AA in D-L diastereomer results in a gap and less symmetrical form of the molecule in the D-L diastereomer causing stronger interaction with the alkyl chains of the reversed phase and thus greater retention time in comparison with the L–L diastereomer.

These observations are in agreement with Marfey's explanation (Marfey, 1984) that H-bond is the most

important feature which brings differences in the free energies of diastereomers owing to its formation/non-formation and for obtaining large Δt_R values (Brückner and Keller-Hoehl, 1990).

(ii) in the L–L and D-L diastereomers of Asp and Glu acids (to be analysed) the respective β- and γ-carboxyl groups probably do not exhibit a different behaviour as exhibited by mono carboxyl amino acids. The relatively higher hydrophilicities in these cases lead to shorter retention times and lower Δt_R values as compared to neutral side chain AAs.

(iii) AAs in diastereomers have high steric hinderance due to side chains and there is almost no rotational freedom, *e.g.*, this is lower for valine having a C^{α}-isopropyl chain as compared to Ala having C^{α}-methyl side chain; these steric factors further facilitate resolution of diastereomers. The models also suggested that the proline ring in the respective diastereomer was perpendicular to the benzene ring leading to steric hinderance in the reagent FDNP–Pro–NH_2.

(iv) all reagents with the formulae FDNP–L-AA–NH**R**, FDNP–L-AA–**OR**, or FDNP–L-Ala–Ala–NH_2 have resolution capacity as diastereomers formed by reaction with DL-amino acids, as in all cases an intramolecular H-bridge can be formed for the L–L diastereomer. This is not the case with reagents having structures FDNP–NH**R**; the moiety **R** alters the electronegativity of the carboxy group in –CONH**R** and –COO**R**, and also the hydrophobicities of diastereomers. The larger Δt_R values are obtained by diastereomers formed by reaction of H–L-Val–OH with FDNP–L-Val–OH since in the resulting L–L diastereomer the –COOH groups of amino acids can form H-bridges to each other.

Fujii et al. (1997a) investigated the separation mechanism and determination of absolute configuration using UV and NMR techniques which are the instrumentations susceptible to conformation.

The UV spectra of the FDAA derivatives of the amino acids (under study) suggested that the stable conformation, including intramolecular H-bonding, between the two nitro groups in the benzene ring and both amino groups of the amino acid and L-alaninamide, is formed as a planar molecule of a three-ring system, like anthracene (Fig. 2). Further, the NMR spectra of L- and D-valine derivatized with FDAA indicated that both α-protons were spatially situated near H-6 of the benzene ring in both L- and D-amino acid derivatives. Thus the resulting conformations of the L- and D-valine derivatives (Fig. 2), in which each substituent (except for the amino groups of Val and L-alaninamide) was oriented perpendicular to the planar molecule of the dinitrobenzene, were stable and predominant in solution. Therefore the FDAA derivative of D-valine had the *cis* (Z) type arrangement of two more hydrophobic substituents of valine and L-alaninamide (the isopropyl group and methyl group, respectively) to the plane of the dinitrobenzene, whereas the FDAA derivative of L-valine had the opposite arrangement (*trans*, (E) type).

Since the resulting conformations of the L- and D-amino acid derivatives were stable their resolution was due to difference in their hydrophobicity, which is derived from the *cis*- or *trans*-type arrangement of two more hydrophobic substituents at both α-carbons of an amino acid and L-alanine amide, so that the FDAA derivative of the *cis* (Z)-type arrangement interacts more strongly with ODS silica gel and has a longer retention time than that of the *trans* (E)-type arrangement. Therefore, the L-amino acid derivative is usually eluted first from the column.

The mechanism gets confirmed with the experimental observation that the retention times of derivatized amino acids with FDAA were dependent on the hydrophobicity of amino acids, e.g., the FDAA derivative of an amino acid, which has a larger difference in hydrophobicity between the α-carboxyl group and the side chain, has a longer retention time and a better resolution. Separation behaviour of FDAA derivatives of amino acid methyl esters and amino compounds without the α-carboxyl

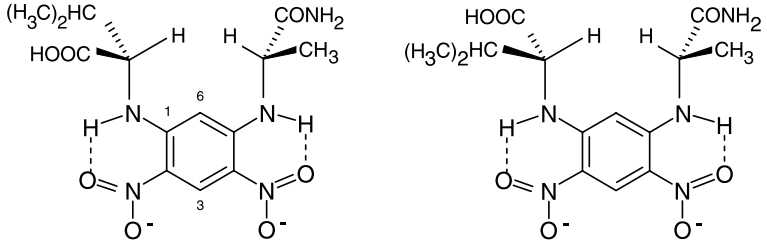

Fig. 2. Plausible conformations of the L-L- and L-D-diastereomers of L- and D-valine with FDAA (Reprinted with permission from Fujii et al., 1997a; Copyright (1997) American Chemical Society)

group, such as 1-phenylethylamine, alanilol and valinol, showed that the retention times of the methyl esters bacame longer than those of the parent amino acids, *i.e.*, the resolution power of amino acid methyl ester decreased in comparison with that of the parent amino acid. Particularly, the FDAA derivative of alanine methyl ester was not resolved, and the serine methyl ester derivatives showed the opposite elution order. The retention times and resolution power of the amino compound derivatives were almost the same as those of the parent amino acids.

The separation mechanism for the primary amino compounds derivatized with FDPEA has been based on a conformation (Fig. 2) investigated by UV and NMR, assisted by the intramolecular H-bonding between the nitro groups and α-amino groups of the target compound and was quite similar to those of the FDLA derivatives of amino acids and primary amino compounds (Harada et al., 1998).

Separation behaviour of certain derivatized amino acids with chiral variants of FDAA in which L-Ala–NH_2 was replaced with L-Val–NH_2, L-Phe–NH_2, L-Ile–NH_2, and L-Leu–NH_2, further supported the mechanism since the retention time became longer, and the resolution became better with the increase of their length of alkyl side chains in the amino acid amides (Fujii et al., 1997a). The derivatized amino acid with the reagent of D-alaninamides showed the completely opposite elution order. These results indicated that the α-carboxyl group of an amino acid was not always essential for the resolution and the separation behaviour could be explained without consideration of intramolecular H-bonding (between the carboxyl of analyte and the carboxamide of the reagent).

Nevertheless, the mechanism discussed by Marfey (1984), Brückner and Keller-Hoehl (1990) and Brückner and Gah (1991), based on different conformations and intramolecular H-bonding, correlates the separation behaviour to differences in the hydrophobicity of the two diastereomers with the D-diastereomer producing a more hydrophobic molecule which would be expected to interact more strongly with the RP column and thus have a stronger retention time.

The main difference in the proposed separation mechanisms can be looked into in terms of the structure of the plausible conformations (Fig. 1b and Fig. 2). Looking to the structure drawn by Marfey (1984) and Brückner and Keller-Hoehl (1990) it is apparent that a 12 member ring due to H-bond formation between carbonyl oxygen of the *meta*-situated L-Ala–NH_2 with the H atom of the carboxyl group of the L- or D-amino acid is equally likely as there is a free rotation between the C^{α}- and amino –N of the amino acid moiety that allows equal chances for the –COOH group to come on either side (and this rotation is not affecting the configuration).

On the other hand, the Fig. 2 (Fujii et al., 1997a) shows that a H-bond between oxygen atom of the nitro group in position 2 of the benzene ring with amino –H of the amino acid (Val, in this case) and another H-bond formation between nitro group at position 4 in the benzene ring and the amino –H of the alanine amide moiety (of the reagent) is possible forming a six membered ring on either side. A six membered ring, nearly planar, on both sides provides a more stable state of the complex (in comparison to a 9 or 12 membered ring). Such a structure is possible for both L- and D-configurations of the amino acid. Thus the two structures shown in Fig. 2 behave as diastereomers due to the difference in steric arrangement of the groups at the stereogenic centre of the α-amino acid (to be analysed). Further, the hydrophobic methyl (of alaninamide) and isopropyl (of valine) groups in D-Val–DNPA are *cis* to the plane of dinitrobenzene and thus interact more strongly with ODS silica gel and have a longer retention time. The FDAA derivatives of D-amino acids have *cis*-type arrangement because in most of the α-amino acids the side chain is more hydrophobic than the carboxylic group. Therefore, FDAA derivative of the L-enantiomer is usually eluted before the corresponding D-isomer. Participation of the nitro groups at hydrogen bonds is supported by the fact that *s*-triazine CDR's, which are considered to be structurally related to MR (Brückner and Strecker, 1992), in general are less effective for the indirect chiral separation of amino acids (Brückner and Wachsmann, 2003).

Thus in both the cases (Figs. 1 and 2), H-bond plays a role in the overall stability of the diastereomeric complex except that the site of H-bond is different. The *three point rule* (Dalgliesh, 1952) proposed for resolution of enantiomers considers H-bond as one of the important factors along with π–π interactions and steric repulsions, between the CSP and one of the enantiomeric forms to distinguish between the two enantiomeric forms. In the application of MR the stationary phase is achiral but the MR being chiral is responsible for diastereomeric formation and the differential interaction of the diastereomers with the ODS causes separation.

6 Applications and comparison with other derivatizing agents

6.1 Amines and non-proteinogenic amino acids

As in the case of α-amino acids, the fluorine atom of MR is rapidly and quantitatively substituted under alkaline conditions by certain racemic benzylic amines, such as (*RS*)-α-methylbenzylamine, α-naphthylethylamine, 1,2,3,

4-tetrahydro-α-naphthylethylamine, 1-methyl-3-phenylpropylamine, and α-methyl-*p*-nitrobenzylamine, and has been used as an effective NMR chiral auxiliary that induced adequate chemical shift non equivalence for the two diastereomers formed; in all cases rapid and accurate determination of diastereomeric composition was achieved (Calmes et al., 1993). The enantiomeric excess of 2-homoarylglycines, during their asymmetric synthesis, via hydrolysis of the diastereomerically pure *N*-phthalyl pantolactonyl esters under acidic conditions, was determined by NMR analysis after derivatization with Marfey's reagent (Calme's et al., 2000).

The reagent has been successful in HPLC separation (Brückner et al., 1988, 1989) of diastereomers of non-proteinogenic α-hydroxymethyl-α-amino acids and α-alkyl-α-amino acids (AAAs) which were also separable as diastereomeric esters by GC (Brückner and Langer, 1991); these diastereomers of AAAs formed with MR, like those of proteinogenic amino acids, showed exceptionally large differences in retention times in HPLC in comparison with other methods such as the formation of diastereomers by derivatization with *o*-phthaldialdehyde and chiral thiols (Brückner et al., 1991b).

A number of (27) racemic α-alkyl-α-amino acids (AAAs) were derivatized with either *o*-phthaldialdehyde (OPA) in combination with *N*-*t*-butoxycarbonyl-L-cysteine (Boc-Cys) or *N*-acetyl-cysteine (Ac-Cys) or with MR. RP-HPLC was successful in resolving all the diastereomers completely, formed by MR, using TEA-phosphate buffers of pH 3.0 (pH 7.2 for acidic AAA) together with acetonitrile at 340 nm. Only 8 or 11 diastereomers formed with OPA/Boc-Cys or OPA/Ac-Cys were resolved respectively using sodium phosphate buffer of pH 7.2 together with acetonitrile and fluorescence detection (Brückner and Zivny, 1993). Thus, derivatization of AAA with MR has been considered to be a highly suitable method for their resolution.

Similarly, MR has been found to be more efficient in comparison to GITC (2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate) as a CDR for HPLC resolution of several β-substituted β-alanines as the k' values for the first-eluting and the second-eluting components were 2–3 times higher, respectively, than those for the GITC derivatives (Péter et al., 2001). The resolution of enantiomers of ring- and α-methyl substituted phenylalanines and phenylalanine amides (12 numbers) has been compared (Péter et al., 2000) using both direct and indirect (derivatization with FDAA and GITC) RP-HPLC methods. The direct separation was carried out on crown-ether-based (Crownpak CR(+) and the teicoplanin-based (Chirobiotic T) columns while the indirect separation was carried out by pre-column derivatization with GITC and FDAA. It was found that Chirobiotic T column was efficient in the separation of ring- and α-methyl substituted phenylalanines but was ineffective for the amides of these analogues. The Crownpak CR(+) column separated ring-substituted phenylalanines and amides, whereas α-methylated analogues were co-eluted. Of the two indirect methods, GITC derivatization was more effective than FDAA derivatization.

Derivatization with FDAA followed by HPLC has been successful, and more favourable in comparison to the GITC derivatives, for separation and identification of enantiomers of β-amino acids possessing the bicyclo[2,2,1]heptane rings (Török et al., 1998) such as *diendo*- and *diexo*-3-amino-bicyclo[2,2,1]heptane-2-carboxylic acids (*diendo*-ABHC and *diexo*-ABHC respectively), and of *diendo*- and *diexo*-3-amino-bicyclo[2,2,1]heptene-2-carboxylic acids (*diendo*-ABHC-ene and *diexo*-ABHC-ene, respectively) (Fig. 3). It is interesting that enantiomers of molecules with axial chirality such as, 2′,1′:1,2;1″,2″:3,4-dinaphthcyclohepta-1,3-diene-6-aminomethyl-6-carboxylic acid containing a free amino group (β^2-bin, Fig. 4) and their N- and/or C-terminal protected derivatives have also been resolved in a similar

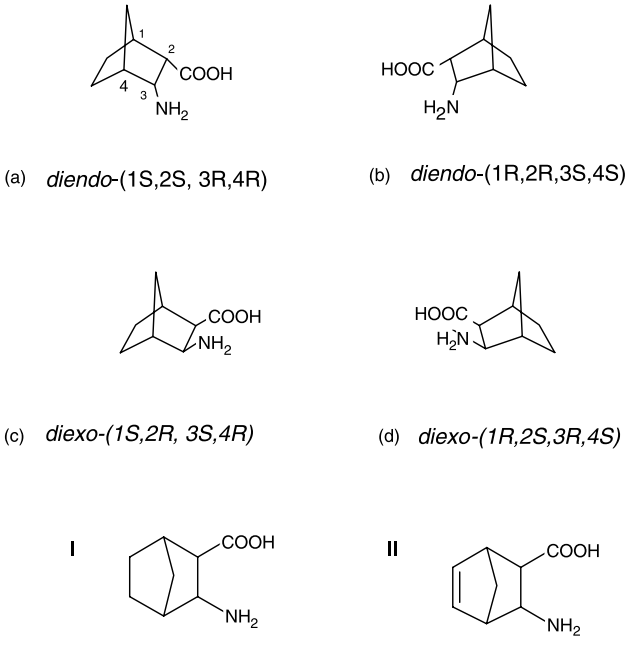

Fig. 3. Structures of four enantiomers of (**I**) ABHC, and (**II**) ABHC-ene; ABHC = 3-amino-bicyclo[2,2,1]heptane-2-carboxylic acids. (**a**) *diendo*-(1*S*,2*S*,3*R*,4*R*) isomer (**b**) *diendo*-(1*R*,2*R*,3*S*,4*S*) isomer; (**c**) *diexo*-(1*S*,2*R*,3*S*,4*R*) isomer (**d**) *diexo*-(1*R*,2*S*,3*R*,4*S*) isomer (Török et al., 1998)

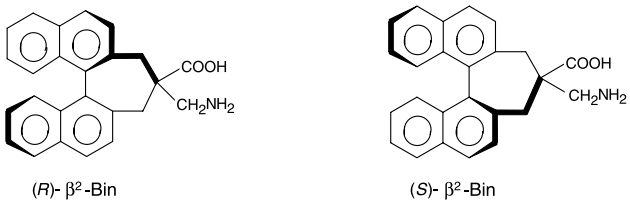

Fig. 4. Enantiomers of β^2-Bin (2′,1′:1,2;1″,2″:3,4-dinaphthcyclohepta-1,3-diene-6-aminomethyl-6-carboxylic acid), the molecules possessing only axial chirality (Török et al., 1999)

manner; for each derivatized compound, different organic (CH_3OH and CH_3CN) and aqueous (0.1% TFA, 0.01 M NaOAc, pH 3.0) solvents were used at different volume ratios (Török et al., 1999).

Shimada et al. (1993) compared the HPLC separation of diastereomers of baclofen (4-amino-3-chlorophenyl butyric acid), a skeletal muscle relaxant administered clinically as a racemic mixture, prepared by derivatization with FDAA, GITC, (+)-1-(1-naphthyl)ethyl isocyanate (NEI), and (+)-1-(9-fluorenyl)ethyl chloroformate (FLEC). The FDAA diastereomers gave the most satisfactory results in the conventional HPLC (MeOH-0.5% KH_2PO_4 (11:8), pH 4.0).

Absolute configuration of an acyclic secondary alcohol using the characteristic functions of FFDNB was determined. The D- and L-secondary alcohol reacted first with FFDNB under mild basic conditions, and L-leucinamide or DL-leucinamide was then introduced into the secondary alcohol-FDNB derivative. The dinitro leucinamide (DLA) derivative so obtained were subjected to ordinary HPLC analysis under reversed-phase conditions. Because the conformations of the resulting alcohol-DLA derivatives were rigidly fixed by the dinitrobenzene plane, the absolute configuration at the asymmetric carbon of the secondary alcohol was deduced by the elution behavior of both of the diastereomers in the HPLC and/or LC/MS (Harada et al., 2000).

6.2 Peptides and amino acids from micro-organisms

The configuration of the amino acids of laxaphycins A and B (cyclopeptides) produced by the tropical marine cyanobacterium *Lyngbya majuscula* was established by HPLC analysis of diastereoisomers formed on reaction of the amino acids with FDAA in the hydrolysates. The results were supplemented with NMR, FAB MS to determine their total structure (Bonnard et al., 1997). Mikkola et al. (2000) used Marfey's reagent to analyse D- and L-amino acids in cyclic lactonic heptalipopeptides. The toxins, isolated from three *Bacillus licheniformis* strains, were connected to a fatal food poisoning and each was found to contain the same six amino acids in the sequence of L-Gln, L-Leu, D-Leu, L-Val, L-Asp, D-Leu and L-Ile.

Contents of D-enantiomers of Ser, Ala, Pro, Gln, Asn, and Phe were determined in various eubacteria, some archaea and some eukaryotes, such as, *Staphylococcus epidermidis*, *Streptococcus pyogenes*, *Thiobacillus ferrooxidans*, *Pyrobaculum islandicum* (Nagata et al., 1998a). Bacterial cells were harvested, dried, and hydrolysed and the resultant hydrolysates were treated with FDAA to form diastereomers of amino acids. The FDAA derivatives were separated by 2D TLC on silica gel 60 plates. The FDAA amino acids recovered from TLC were analyzed by HPLC for the resolution of D- and L-isomers.

FDLA instead of FDAA with LC/MS using frit-FAB or ESI as the interface has been applied non empirically to determine the absolute configuration of certain unusual amino acids, such as, *N*-methylalanine (MeAla), and homotyrosine (Hty) in the anabaenopeptins-A and -B produced by cyanobacterium *Anabaena flos-aquae* NRC 525-17 (Harada et al., 1995), and constituent α-amino acids like Glu-γ-carbonyl-γ-aldehyde, pentahomoserine and proline in aeruginopeptin 228A (Harada et al., 1996), while β-methyl aspartic acid (β-MeAsp), Ala, Leu, Arg, Glu in microcystin LR, both produced by cyanobacterium *Microcystis aeruginosa* M228 (Harada et al., 1996; Fujii et al., 1997b). Marfey's method has been used to determine the absolute configurations of the constituent amino acids, (*viz.*, D-glutamine, glycine, L-phenylalanine, D-*allo*-isoleucine, 2 mol of L-proline, and a novel β-amino acid moiety, (2*S*,3*R*,5*R*)-3-amino-2,5-dihydroxy-8-phenyl-octanoic acid) of nostophycin, a cyclic peptide, isolated from cyanobacterium *Nostoc* sp. strain 152. The separation of the L- and DL-FDLA derivatives was performed on an ODS column maintained at 40°C using acetonitrile-water containing 0.01 M TFA as the mobile phase under a linear gradient elution mode (acetonitrile, 30–70%, 40 min) at a flow rate of 0.2 mL/min (Fujii et al., 1999).

Besides, structural characterization including absolute configuration of constituent amino acids of the antibiotic peptide colistin (CL), produced by *Bacillus polimyxa* var. *colistinus* were carried out using Frit-FAB LC/MS, MS/MS, and FDAA for HPLC analysis of amino acids (Ikai et al., 1998). Substances suspected to be new microcystins (cyclic peptides), in extracts of various algae and cyanobacteria occuring world wide in marine waters and lakes, were hydrolysed and derivatised with FDLA followed by enantioselective analysis of amino acid derivatives by LC-ESI-MS (Dahlmann et al., 2003).

Assignment of absolute stereochemistry to aspergillicin-A, a new depsipeptide isolated from *Aspergillus carneus*, was achieved by HPLC analysis of the Marfey's derivatised acid hydrolysate. Aspergillicin A was found to comprise L-Val, *N*-Ac-L-Thr, D-Ile, L-Pro (×2) and *N*-Me-L-Tyr-*O*-Me. Analysis of the Marfey's derivatives on a chiral HPLC column (Phenomenex Chirex, urea type 3010) differentiated D-Ile from D-*allo*-Ile, or L-Ile from L-*allo*-Ile that could not be differentiated with ESIMS and NMR (Capon et al., 2003). Determination of amino acid configuration of a new cyclohexadepsipeptide, isolated from a cell extract of entomopathogenic fungus *Hirsutella kobayasii* BCC 1660, was carried out using FDAA and RP HPLC but resolution of L-isoleucine and L-*allo*-isoleucine was achieved using a chiral column (Vongvanich et al., 2002).

6.3 Cysteine residue in peptides

Regardless of the thiol protecting groups used in the synthesis of cysteine peptides, racemization of this residue is a serious problem in both liquid and solid phase peptide synthesis. The highly reactive –SH group of cysteine is prone to oxidation, elimination and modification by electrophilic compounds. The chiral analysis of hydrolyzed csyteine peptides is very difficult as the peptides containing cysteine epimerize during hydrolysis. The –SH group forms thiazoline adduct with *N*-adjacent residues under acidic conditions. Derivatization of the –SH group, prior to acid hydrolysis, with 4-vinylpyridine protected the cysteine from other reactions, prevented thiazoline formation and suppressed racemization and enantiomeric resolution of D,L-S-β-(4-pyridylethyl)cysteine was achieved (Siedler et al., 1996) by CZE with crown ethers or by GC on chiral glass capillary column. Jacobson et al. (1998) derivatized S-pyridylethylated cysteine with FDAA and achieved baseline separation of the diastereomers of cysteine in somatostatin analogues with HPLC (Buffer A: 0.1% TFA in water; buffer B: 0.1% TFA in MeCN, gradient 11% to 66% in 50 min, flow rate 1 ml/min). The reaction with S-(4-pyridylethyl)cysteine proceeds in a manner similar to other non-derivatized amino acids. The results showed that this derivatization protected chirality of cysteine during hydrolysis.

Szabó et al. (2001) compared racemization studies, using Marfey's method, and adopting the approach of separating epimeric mixtures of oxytocin and other peptides containing cysteine or cystine. Various CDRs used include, OPA with *N*-isobutyryl-L-cysteine additive, GITC, 2,3,4,6-tetra-*O*-benzoyl-β-D-glcopyranosyl isothiocyanate (BGIT), FDAA, FDVA. Both GITC and BGIT increased hydrophobocity of cysteic acid, due to their four *O*-acyl groups, and were found suitable for the separation of enantiomers but GITC resulted better separation than BGIT. FDAA was suitable for separation of homocysteic acid (which has one more $-CH_2$) while FDVA was suitable for the separation of L- and D-cysteic acid. Absolute configuration of microcyclamide (a peptide) possessing thiazole containing amino acids, and for two other naturally occurring peptides, was determined using FDLA for derivatization followed by HPLC. Flash hydrolysis was introduced to depress racemization. An additional advantage was that the labile amino acids such as tryptophan and methionine sulfoxide could be detected, during acid hydrolysis, in the intact form (Fujii et al., 2002).

6.4 Complex mixtures

Pre-column derivatization with the reagent has been used to quantify the common 19 DNPA-L-amino acids (Kochhar and Christen, 1989; Kochhar et al., 2000). Acid hydrolysis of peptides and the use of MR has generated accurate results with little racemization of amino acid isomer residues when Goodlett et al. (1995) resolved 38 DNPA-D- and DNPA-L-amino acids in one chromatographic separation by conventional RP HPLC using ammonium formate (10 mM, pH 5.2) mobile phase consisting of 1% methanol in both A and B, with 5% acetonitrile in A and 60% acetonitrile in B (linear gradients started with 0% B and finished 100% B in 45 min, flow rate 1 ml/min). Alternatively, the TFA mobile phase (0.05%), with the same concentrations of methanol and acetonitrile as used in ammonium formate buffer, provided better resolution of histidine diastereomer pair but those of Gln, Pro, and Ser were not resolved. The L-isomer eluted before D- in all the cases (Table 1). The optical purity of a synthetic decapeptide was established by Marfey's method when ten FDAA derivatives were separated and distinguished as L-Glu, D-Glu, L-Ala, L-Pro, L-Ile, L-Tyr (mono- and *bis*-derivatives), and L-cyclohexyl amine, in a single chromatographic run using sodium acetate (0.04 M, pH 5.3) with acetonitrile-water (10:90) as mobile phase A and acetonitrile-water (50:50) as mobile phase B (B'Hymer, 2001). Free solution capillary electrophoresis (FSCE) and miscellar electrokinetic capillary chromatography (MECC) in combination with L- and D-Marfey's reagent has been used for analysis of racemic amino acids (Ala, Asp, Glu, Leu, Phe and Trp), isomers of a di-peptide (L-D < L-L < D-D < D-L) and a tri-peptide (Tran et al., 1990).

Table 1. HPLC separation of 19 common D- and L-amino acid pairs with FDAA

Amino acid	Retention time			
	Ammonium formate[1]		TFA[2]	
	L	D	L	D
Ala	10.93	13.66	18.34	19.98
Arg	10.29	11.17	14.45	15.38
Asn	7.88	9.63	13.93	14.21
Asp	7.98	9.85	15.88	16.33
Cys	10.89	12.56	11.66	13.08
Glu	9.42	11.35	17.11	17.77
Gln	8.97	1.03	14.99	14.99
His	9.08	9.23	11.85	13.37
Ile	17.01	20.94	25.36	28.03
Leu	7.58	21.28	25.64	28.10
Lys	9.67	10.57	13.98	15.03
Met	14.54	17.98	22.38	24.62
Phe	18.33	21.23	25.95	27.85
Pro	11.57	13.82	19.12	19.12
Ser	8.48	9.93	14.80	14.80
Thr	9.01	12.28	15.54	17.57
Trp	18.37	20.52	25.20	26.52
Tyr	13.34	15.67	18.05	20.73
Val	14.29	18.22	22.88	25.49

UV, 340 nm
[1] Ammonium formate (10 mM, pH 5.2) mobile phase consisting of 1% methanol in both A and B with 5% acetonitrile in A and 60% acetonitrile in B (linear gradients started with 0% B and finished 100% B in 45 min, flow rate 1 ml/min)
[2] TFA mobile phase (0.05%), with the same concentrations of methanol and acetonitrile as used in ammonium formate buffer
[Ref. Goodlet et al. (1995)]

6.5 Evaluation of racemizing characteristics

Racemization of amino acids is known to occur concomitent with acid hydrolysis. In order to determine the chiral purity of amino acids in a peptide, racemization must be corrected or circumvented. Accurate correction factors are difficult to obtain because racemization rates depend on the location of an amino acid in a peptide and differ between individual free amino acids. Methods that label amino acids, as racemization occurs, are preferred over correction factors because they offer an *in situ* control. Goodlett et al. (1995) circumvented racemization by hydrolysing peptides in ^{2}HCl/[$^2H_4$]acetic acid (1:1); amino acids were then derivatized with FDAA and analysed by LC-ESI-MS on a RP column. This method allowed the original chiral purity of each amino acid to be determined. Any amino acid that racemizes during hydrolysis will be labeled with one deuterium at the α-carbon. Thus racemized amino acid could be distinguished from the non-racemized by an increase of one atomic mass unit using MS. At unit resolution a quadrupole mass spectrometer can distinguish between two ions that differ by only one m/z value and this would allow non-racemized amino acid to be distinguished from the amino acid that racemized in an environment of deuterium. However, the method requires equipment that is not readily accessible and has high equipment and maintenance costs.

Using MR, Adamson et al. (1992) developed a chromatographic assay to quantitate racemization occuring during attachment of protected amino acids to solid supports used in peptide synthesis. Acidolytic cleavage of deprotected amino acids from supports and subsequent derivatization with MR gave diastereomers (Fig. 5) separable by RP HPLC using *aq* acetonitrile

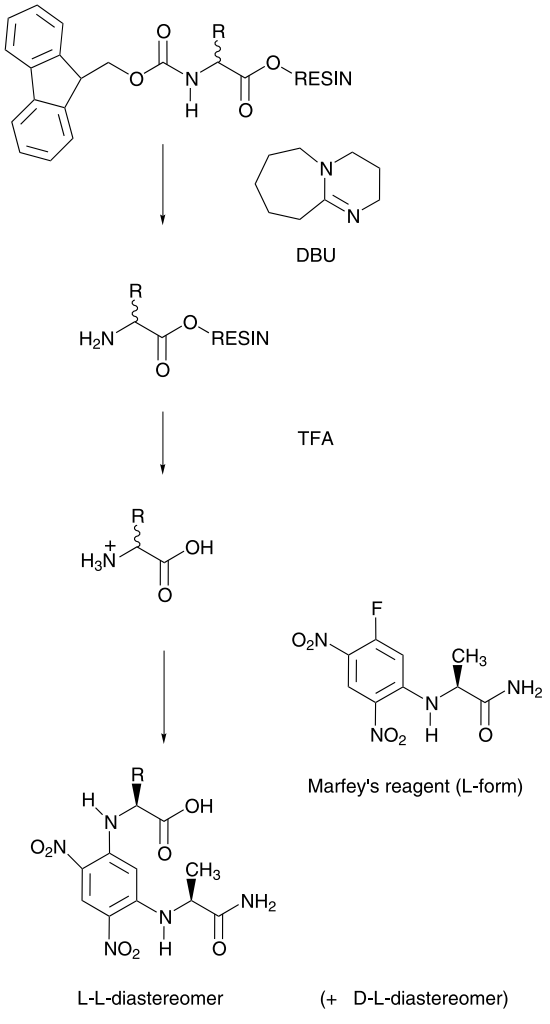

Fig. 5. D, L-amino acid quantitation on solid phase peptide synthesis support (*DBU* 1,8-diazabicyclo[5,4,0]undec-7-ene); (Adamson et al., 1992, modified)

(2 ml/min, 10–60% acetonitrile over 50 min, or 5–20% acetonitrile in 70 min; or isocratic conditions, 8% acetonitrile in 20 mM sodium acetate buffer, pH 4.0); 0.1% TFA was maintained throughout elution in the case of gradients.

Szókán et al. (1988, 1989) extended the method to the analysis of biologically active peptides, mixtures of all common protein amino acids, their amino- and carboxy-protected derivatives, branched polypeptides based on polylysine, and endothiopeptides and to the detection of racemization of peptides. Pre column derivatization with MR was used (Szabó et al., 2000; Szókán et al., 1994) to monitor enantiomeric impurities and racemization in various amino-, carboxy- and/or side chain protected amino acid derivatives using C18 column (ODS-Hypersil). High α and R_s values were achieved, and in majority of cases L- eluted before the D-isomer. The chromatographic patterns/behaviour were reported to be in good accordance with Brückner et al. (Brückner and Keller-Hoehl, 1990; Brückner and Gah, 1991) explanation and with NMR results of Fujii et al. (1997a, b). Somali et al. (1992) determined optical purity of a number of amino acid amides by derivatizing them with MR followed by HPLC (MeOH-NaOAc buffer, pH 4).

When the same amino acid occurs repeatedly in the peptide sequence, it would be desirable to determine the chirality of each residue as a function of position. This, unfortunately, cannot be done by determining the enantiomeric purity of the PTH-amino acid obtained in the course of Edman degradation, since these generally undergo racemization. Scaloni et al. (1991) used FDNPA instead of FDAA in the protocol of subtractive Edman procedure to determine the sequence of amino acid residues with concomitant identification of their chirality at a nanomolar level. 14 pairs of enantiomeric amino acids from both natural and small synthetic peptides were analyzed using TEAP buffer (40 mM, pH 2.2). Racemization of L- to the D-isomer was found to be in the range of 1 to 3% after 24 hr of hydrolysis. The method was also successful with MR when the amino acid derivative gave the deaminated version during the acid hydrolysis step. Later, a fully automated system permitting analysis of fifteen amino acid pairs utilizing the commercially available MR was reported (Scaloni et al., 1995).

6.6 Biological fluids and samples

HPLC after derivatization with FDAA has routinely been carried out for determination of D-serine in the cerebrum and cerebellum of mutant mouse ddY/DAO$^-$ to establish the involvement of D-amino acid oxidase in elimination of free D-serine in the brain (Nagata, 1992), in the *bus* mouse (Nagata et al., 1997) and in the brains of SAMP8 and SAMR1 (senescence accelaration-resistant mouse) strains (Nagata et al., 1998b), determination of D- and L-serine (D/L ratio being 0.086) in normal and Alzheimer human brain (Nagata et al., 1995) and D-and L-serine in extracts of vertebrate brains to determine distribution levels of free D-serine in terms of D-amino acid oxidase activity (Nagata et al., 1994a), to resolve and determine the enantiomers of Ala, Ser, Pro, Glu, and Asp in serum, liver, kidney and brain of ddY/DAO$^-$ mice (Nagata et al., 1994b; Nagata and Kubota, 1993), determination of D/L ratio of 12 neutral free amino acids in human plasma samples from patients with renal diseases and from normal subjects (Nagata et al., 1992a). In most of these reports from Nagata and co-workers 2D TLC has been performed using *n*-butanol-acetic acid-water (3:1:1, v/v) in the first dimension and phenol-water (3:1, v/v) in second dimension followed by scrapping and extracting of the spots with methanol-water (3:1, v/v) prior to HPLC resolution of diastereomers for recognising D- and L-enantiomers (Nagata et al., 1992b) and also applied to demonstrate the presence of D-enantiomers of Ala, Pro and Ser in mouse kidney.

MR was shown to be a viable CDR for enantiomeric determination of amphetamine (AMP) and methamphetamine (METH) in human urine. The enantiomers were isolated from urine by solid phase extraction and diastereomers were formed with FDAA. HPLC in a water-methanol mobile phase established a detection limit of 0.16 mg/L urine for each enantiomer. The method gave results comparable with those obtained from the more sensitive liquid–liquid extraction (LLE) and derivatization with (−)-1-(9-fluorenyl)ethyl chloroformate (FLEC) and fluorometric detection (Foster et al., 1998). Analysis of serine isomeric composition in purified urine sample of a ddY/DAO$^-$ mouse, lacking D-amino acid oxidase (Asakura and Konno, 1997), and separation and quantitation of D- and L-phosphoserine in rat brain (Goodnough et al., 1995) was carried out by FDAA-derivatization reaction, followed by HPLC. It is worth noting in this context that MR gives larger resolution and is much less expensive in comparison to FLEC.

7 Direct resolution

Pre-column derivatization with MR supported by the efficient RP-HPLC separation of diastereomers has acquired a great popularity and routine usage in the analysis of

optical purity and determining absolute configuration of the consitituting amino acid residues in peptides and all other different amino acids. It has found applications in many diverse areas of biochemical research including determination of substrates and products in enzymic reactions of amino acids.

MR has also been used for direct resolution of enantiomers. Two CSPs were prepared by covalent bonding of aminopropylsilica (APS) to (i) MR (FDAA) followed by end capping of underivatised amino groups of APS with trifluoroacetic anhydride, and (ii) by reaction of APS with 1-fluoro-2,4-dinitrophenyl-5-L-phenylalanine *tert*-butyl ester, followed by cleavage of *tert*-butyl ester by TFA and end capping of underivatized amino groups of APS with *n*-butyryl chloride (Brückner and Leitenberger, 1996). The two CSPs were found to be suitable for the chiral resolution of 2,4-DNP- and 3,5-dinitrobenzoyl-DL-amino acid esters by liquid chromatography using mixtures of *n*-hexane and *iso*-propanol as eluents. The resolution could be explained on *three point rule* (Dalgliesh, 1952) involving $\pi-\pi$ donor-acceptor interactions, dipole-attraction-repulsion (stacking), H-bonding, and van der Waals' attractions together with steric interactions.

8 Conclusion

It is evident that amino acid analysis following reaction with Marfey's reagent permitted very sensitive detection of racemization and in several instances, revealed higher racemization than shown by CPA/MALDI-MS. Information could be obtained even if the synthesis failed.

Though MR has many inherent disadvantages of an indirect method of enantiomeric resolution or purity determination it gains advantages over several other derivatizing reagents and many chiral stationary phases since these CSPs are not as durable. The structural features of MR provide a flexibility and possibility to increase its hydrophobicity (thus increasing α and R_s of the derivatives), by replacing the chiral selector Ala-NH_2 of the reagent with other suitable moieties and make the method quite flexible for resolution and quantitation of DL-amino acids in different situations. It further establishes utility and importance of MR and renewed interest.

The separation mechanism involves the formation of H-bridge to provide a stable conformation which is responsible for elution behaviour (L-isomer eluting before D-), and the α-amino group is essential for the resolution of both diastereomers. Elution order of Orn has been opposite (D- followed by L-isomer) and the efforts to elucidate the conformation by X-ray were not successful. Nevertheless, the final structures of conformations explaining the site of H-bonding (and elution order) in all the cases may be elucidated with X-ray investigations. Structural fine tuning of the CSPs developed from covalent bonding of MR or its analogs with APS etc will enable a great variety of CSPs suitable for direct resolution too.

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