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Review

Application of chiral derivatizing agents in the high-performance liquid chromatographic separation of amino acid enantiomers: A review

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Abstract

The past 20 years has seen an explosive growth in the field of chirality, as illustrated by the rapid progress in the various facets of this intriguing field. The impetus for advances in chiral separation has been highest in the past decade and this still continues to be an area of high focus. This paper reviews indirect separation approaches, *i.e.* derivatization reactions aimed at creating the basis for the chromatographic resolution of biologically and pharmaceutically important enantiomers, with emphasis on the literature published in the last 12 years. The main aspects of the chiral derivatization of amino acids are discussed, *i.e.* derivatization on the amino group, transforming the molecules into covalently bonded diastereomeric derivatives through the use of homochiral derivatizing agents. The diastereomers formed (amides, urethanes, urea, thiourea derivatives, etc.) can be separated on achiral stationary phases. The applications are considered, and in some cases different derivatizing agents for the resolution of complex mixtures of proteinogenic D,L-amino acids, non-proteinogenic amino acids and peptides/amino acids from peptide syntheses or microorganisms are compared.

Keywords: HPLC; Amino acids; Proteinogenic; Non-proteinogenic; Enantiomeric resolution; Indirect methods; Diastereomer formation; Chiral derivatizing agent (CDA)

Contents

Abbreviations: AITC, 2,3,4-tri-*O*-acetyl-α-D-arabinopyranosyl isothiocyanate; APOC, 1-(9-anthryl)-2-propyl chloroformate; APPI, 1-acetoxy-1-phenyl-2-propyl isothiocyanate; BGIT, 2,3,4,6-tetra-*O*-benzoyl-β-D-glycopyranosyl isothiocyanate; Boc, *tert*-butyloxycarbonyl; BTCC, *N*-(*tert*-butylthiocarbamoyl)-L-cysteine ethyl ester; Bzl, benzyl; CDA, chiral derivatizing agent; CDITC, *N*-[(2-isothiocyanato)-cyclohexyl]-6-methoxy-4-quinolinylcarboxamide; CSP, chiral stationary phase; DANI, 1,3-diacetoxy-1-(4-nitrophenyl)-2-propyl isothiocyanate; DBD-PynCS, 4-(3-isothiocyanatopyrrolidin-1-yl)-7-(*N*,*N*-dimethylaminosulfonyl)-2,1,3-benzoxadiazole; DDITC, *N*-3,5-dinitrobenzoyl-*trans*-1,2-diaminocyclohexane isothiocyanate; DNFB, 2,4-dinitrofluorobenzene; ESI-MS, electrospray ionization mass spectrometry; FDAA, 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide; FDIA, 1-fluoro-2,4-dinitrophenyl-5-L-isoleucine amide; FDLA, 1-fluoro-2,4-dinitrophenyl-5-L-leucine amide; FDPA, 1-fluoro-2,4-dinitrophenyl-5-L-phenylalanine amide; FDPEA, 1-fluoro-2,4-dinitrophenyl-5-(*R*,*S*)-phenylethylamine; FDVA, 1-fluoro-2,4-dinitrophenyl-5-L-valine amide; FLEC, 1-(9-fluorenyl)ethyl chloroformate; Fmoc, 9-(fluorenylmethyl)oxycarbonyl; GAL*f*, 2,3,4,6-tetra-*O*-benzoyl-β-D-galactofuranosyl isothiocyanate; GATC, 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl isothiocyanate; GITC, 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate; IBDC, *N*-isobutyryl-D-cysteine; IBLC, *N*-isobutyryl-L-cysteine; MNB-COOH, 2-β-naphthyl-2-methyl-1,3-benzoxadiazole-4-carboxylic acid; NAB-C, 4-(6-methoxy-2-naphthyl)-2-butyl chloroformate; NAC, *N*-acetyl-L-cysteine; NAP, *N*-acetyl-D-penicillamine; NBD-PynCS, 4-(3-isothiocyanatopyrrolidin-1-yl)-7-nitro-2,1,3-benzoxadiazole; NCA, *N*-carboxy anhydride; NIFE, *N*-(4-nitrophenoxycarbonyl)phenylalanine methoxyethyl ester; OPA, *o*-phthalaldehyde; PDITC, *N*-[2-isothiocyanatocyclohexyl]pivalinoyl amide; TATG, 1-thio-β-D-glucose tetraacetate; TBMB-COOH, 2-*tert*-butyl-2-methyl-1,3-benzoxadiazole-4-carboxylic acid; Trt, trityl; Z, benzyloxycarbonyl; UNCA, urethane-protected amino acid *N*-carboxy anhydride.

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1. Introduction

Since the discovery of optical isomerism by Pasteur, the importance of chirality with respect to biological activity has been clearly recognized. The physiological environment within a living organism is chiral, and the biological activities of enantiomeric forms of molecules can differ dramatically. With the exception of glycine, all of the 20 common α-amino acids have a chiral carbon atom adjacent to the carboxyl group. This chiral center allows the existence of a pair of enantiomers.

Some 20 genetically encoded amino acids comprise the building blocks of proteins, which are the most important constituents of all living systems. Multicellular organisms are usually based on L-amino acids. The racemization of the optically active amino acids in the metabolically stable proteins of living systems may take place in dilute acid or base or at neutral pH. The preparation of enantiomerically pure substituted analogs of amino acids is a challenging task that requires accurate analytical methods with which to determine enantiomeric excesses during asymmetric syntheses. Improved synthetic procedures and analytical technologies provide the desired sequence and purity of synthetic peptides, little or no concern generally being devoted to stereoisomeric purity. It is often assumed that racemization does not occur or need not be examined. However, it is well known that the L and D forms of peptides can differ significantly with respect to stability and biological activity. The examination of purity or racemization remains an analytical challenge. Evaluation of the degree to which racemization occurs in the peptides produced is regularly required.

The separation of enantiomeric isomers of amino acids or molecules containing chiral centers has attracted intense interest within the pharmaceutical industry for the past 20 years, and for much longer as concerns biochemical studies. Two main strategies have evolved for the separation of amino acids and other chiral compounds: an *indirect method*, based on the formation of diastereomers by the reactions of amino acids with a chiral derivatizing agent (CDA) and separation of the diastereomeric derivatives on an achiral stationary phase; and a *direct method*, based on the formation of diastereomers on a chiral stationary phase (CSP) or with a chiral selector on an achiral stationary phase in a mobile phase.

The indirect method is an efficient technique for the enantioseparation of amino acids. The advantages and disadvantages of its application are listed in Table 1. The advantages include the commercial availability of a large number of CDAs and a relatively wide choice of chromatographic conditions. The enantiomer molecule and the CDA must possess easily derivatizable and compatible functional groups. The reaction should be comparatively fast, as otherwise a difference in the rate of formation of the diastereomers may cause kinetic resolution. However, it is essential that the chiral derivatization reaction should proceed quantitatively for both enantiomers, and that racemization should not occur. Resolution via diastereomer formation is usually improved when bulky groups are attached to the chiral centers. Furthermore, if the chiral purity of the CDA is not known and/or is not taken into consideration, the chiral purity of the amino acid will not be determined precisely. Thus, the indirect method is not suitable for the analysis of amino acid enantiomers in a standard sample or in pharmaceutical preparations, where a low amount of antipode (at a level of 0.1 or 0.05%) is to be determined. Furthermore, it is not suitable for preparative purposes. However, it is suitable for the trace analysis of amino acid enantiomers in complex matrices such as biological samples because of the introduction of a highly sensitive UV–vis or fluorescence tag with the CDA.

For determination of chiral amino acid composition of synthetic peptides or biological matrices, peptides are hydrolyzed and the liberated amino acids are then converted to diastereomers by derivatization with different CDAs. To differentiate racemization occurring in the course of peptide synthesis or hydrolysis, hydrolysis in deuterated acid solvent and MS analysis are sometimes necessary. Peptide hydrolysis in deuterated acids circumvents the problem by labeling each amino acid that racemizes with one deuterium at the α-carbon.

The *direct method* based on the application of a CSP is divided into two categories: one is the direct enantioseparation of underivatized amino acids on a suitable CSP, but derivatization with an achiral reagent before the enantioseparation is another choice if the aim is to achieve better separability or higher detection sensitivity. However, in this case it is important that the derivatization should proceed completely in both enantiomers and that racemization should not occur. The direct method with chiral mobile phase additives has the same drawback with regard to the chiral purity of the chiral selectors.

The derivatization of enantiomers through the use of homochiral reagents to form diastereomeric derivatives separable on achiral columns was the first widely used general method in the chiral analysis of drugs and related materials. Following the introduction of new chromatographic and capillary electrophoretic (CE) techniques, the importance of enantioseparations based on covalent chiral derivatization has naturally decreased to some extent. However, this general method is still a method of choice that is widely used, especially in HPLC. The reasons for this are the large number of commercially available homochiral reagents and well-established reactions leading to diastereomeric derivatives with excellent separation

Table 1
Comparison of advantages and disadvantages of indirect and direct methods

Indirect method	Direct methods
Advantages	
1. Good chromatographic properties of derivatives	1. The purity of the chiral selector is not critical
2. Elution sequence predictable	2. Similar molar absorptivities of enantiomers
3. Good chromophoric or fluorophoric properties of the reagent (enhanced sensitivity can be achieved)	3. Absence of racemization and kinetic resolution
4. Low cost of achiral columns	4. Racemates without functional groups are separable
5. Method development is simple	5. Preparative applications are available
6. Selectivity can be increased (better separation is often achieved than with a direct method)	6. Change of temperature may be favorable
7. Possibility of appropriate selection of the elution sequence	7. Simple preparation of analytes and simple chromatographic runs
Disadvantages	
1. The purity of the CDA is critical	1. The theoretical plate number of the CSP is small
2. The molar absorptivities of the diastereomers may differ	2. Slow kinetics of desorption
3. The possibility of racemization	3. The elution sequence is not clear
4. The possibility of kinetic resolution	4. No universal column exists
5. The excess of reagent and side products may interfere with the separation	5. CSPs are very sensitive to chromatographic conditions (rather difficult and lengthy method development)
6. Preparative application is restricted	6. High cost of chiral columns
7. Derivatization may be time-consuming	

Note: Much of the material in this Table is from B'Hymer et al. [29].

and detection possibilities allowing tailor-made separations on inexpensive, achiral columns, *i.e.* in possession of the *R* and *S* forms of the reagent, it is possible to have the peak of the enantiomeric impurity eluting before the main peak.

This review deals with one mode of derivatization outlined in the preceding section: the formation of covalently bonded diastereomers, as the basis for separation on achiral columns. Only HPLC derivatization will be discussed, with the omission of analogous procedures for chiral GC, TLC, SFC and CE separations. A further restriction is that only the pharmaceutical and biological aspects of chiral HPLC derivatization on amino group of amino acids will be dealt with.

Even in this restricted field, the limitations of this section do not permit us to cover the extremely large number of publications from the beginning. Earlier information on this subject may be found in the original papers cited in numerous reviews and monographs of either a general or a more specialized character, some representative examples of which are presented in Table 2. These important reviews are good sources for the literature on general chiral HPLC derivatization.

The recent progress covered in this review concerns the period 1995–2006. The relatively large number of papers published since 1995 on new applications of previously described derivatization reagents and on new reagents is an indication that this branch of chiral analysis continues to be of importance up to the present day.

2. Covalent enantiomeric derivatization of the amino group

Many biochemically important compounds, such as amino acids, biogenic amines and drugs, have at least one amino functional group in their structure. The tagging reactions of primary and secondary amines have been extensively investigated. The major types of reactions for chiral amines, involving amino acids, are based on the formation of amides, carbamates, ureas and thioureas. The reactions with acid chloride and chloroformate reagents proceed rapidly to furnish the corresponding amides and carbamates. Another mode of amide formation is the reaction with *N*-succinimidyl ester. This type of reagent is fairly stable and can be used in aqueous media. Chiral isocyanates and mainly isothiocyanates are good labels with which to produce stable ureas and thioureas. The acid halides are also good labels, because the reactions proceed under mild conditions. However, since the reagent readily undergoes hydrolysis with water in the sample solution, contamination with water in the medium should be avoided. *o*-Phthalaldehydes (OPAs) with chiral thiols give highly fluorescent adducts with the primary amino groups of amino acids, ensuring a very low detection limit in enantiomer analysis. CDAs developed earlier are still in use for different new applications. Table 3 lists all the derivatizing agents discussed in this paper.

2.1. Derivatization with activated carboxylic acids and their derivatives

Some of the reagents and reactions in this section merit special attention. Nishida et al. [33] synthetized various fluorescent reagents possessing 1,3-benzodiazole-4- and -5-carboxylic acids. The reagent with a carboxylic group at position C-4 exhibited higher separation ability toward the original enantiomers than the C-5 isomers. Among the fluorescent reagents, 2-*tert*-butyl-2-methyl-1,3-benzoxadiazole-4-carboxylic acid [(*S*)-TBMB-COOH] and 2-β-naphthyl-2-methyl-

Table 2
Titles of selected reviews and monographs on chromatographic enantioseparations

Number	Title	Reference
1	Resolution of optical isomers by gas and liquid chromatography	[1]
2	Resolution of amino acids as diastereomeric derivatives	[2]
3	Resolution of optical isomers by liquid chromatographic techniques	[3]
4	Chromatographic separations of stereoisomers	[4]
5	Chromatographic chiral separations	[5]
6	Indirect separation of enantiomers by liquid chromatography	[6]
7	Indirect chromatographic methods for resolution of drug enantiomers	[7]
8	Chromatographic enantioseparation: methods and applications	[8]
9	Chiral separations	[9]
10	Chiral derivatization	[10]
11	Enantiomeric derivatization	[11]
12	Enantiomeric derivatization	[12]
13	Automated determination of amino acid enantiomers using derivatization with 1-(9-fluoroenyl)ethyl chloroformate and reversed-phase liquid chromatography	[13]
14	Derivatization for chromatographic resolution of optically active compounds	[14]
15	Indirect methods for the chromatographic resolution of drug enantiomers. Synthesis and separation of diastereomeric derivatives	[15]
16	A practical approach to chiral separations by liquid chromatography	[16]
17	Enantiomeric derivatization for biomedical chromatography	[17]
18	Analytical chemistry and biochemistry of D-amino acids	[18]
19	Chiral separations, application and technology	[19]
20	Recent progress in chromatographic enantioseparations	[20]
21	Derivatization of peptides for their determination by chromatographic methods	[21]
22	Chiral derivatization reagents in the bioanalysis of optically active drugs with chromophore-based detection	[22]
23	Handbook of derivatization reactions for HPLC	[23]
24	Modern derivatization methods for separation sciences	[24]
25	Chiral derivatization	[25]
26	Liquid chromatography	[26]
27	Pharmaceutical and biomedical applications of enantioseparations using liquid chromatographic techniques	[27]
28	Resolution of chiral drugs by liquid chromatography based upon diastereomer formation with chiral derivatization reagents	[28]
29	Marfey's reagent: past, present, and future uses of 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide	[29]
30	Marfey's reagent for chiral amino acid analysis: a review	[30]
31	Evaluation of experimental strategies for the development of chiral chromatographic methods based on diastereomer formation	[31]
32	What's news in chromatographic enantioseparations	[32]

1,3-benzoxadiazole-4-carboxylic acid [(*S*)-MNB-COOH] were applied for the enantioseparation of amino acids by reversed-phase HPLC [34–36]. (For the structures of the derivatizing agents see Fig. 1.) Although the derivatization conditions for amino acids were relatively mild, these reagents required activation to the acid halide, such as –COCl and –COF, before coupling. The resulting derivatives provided high fluorescence intensity at 370–380 nm (excitation at 310 nm). The utility of (*S*)-TBMB-COOH has been demonstrated in applications to determine enantiomeric amino acids, acyl-*sn*-glycerols, glycosyl-*sn*-glycerols and other chiral alcohols and amines [37]. The symmetrical anhydride of *N*-ethoxycarbonylvaline ((Eoc-Val)$_2$O) was applied as CDA for the derivatization and separation of Ala, Val, Leu, Phe and Ser (Bzl) enantiomers. Good separations were also achieved, except for Ser, using the anhydride of *N*-ethoxycarbonyl-Phe ((Eoc-Phe)$_2$O) [38]. The enantiomers of Arg and His in a mixture of amino acids were determined when the reaction products were first fractionated by a simple extraction. Brückner and Lüpke [39] developed highly reactive, urethane-protected, L-α-amino acid *N*-carboxy anhydrides (UNCAs): Boc-Phe-NCA, Boc-Asn(Trt)-NCA, Fmoc-Lys(Boc)-NCA, Fmoc-Met-NCA, Fmoc-Ala-NCA, Z-Ala-NCA and Z-Leu-NCA. With a sodium borate buffer and acetonitrile as solvent, the derivatization of proteinogenic amino acids was complete in 3.5 min at room temperature and the resulting diastereomeric, *N*-protected dipeptides were separated on an octadecylsilica stationary phase. *N*-α-Fmoc-L-Leu-*N*-carboxy anhydrides, *N*-α-Fmoc-D-Leu-*N*-carboxy anhydrides, *N*-α-Fmoc-L-Ala-*N*-carboxy anhydrides, *N*-α-Fmoc-L-Val-*N*-carboxy anhydrides and *N*-α-Fmoc-L-Phe-*N*-carboxy anhydrides (Fmoc-L-AA-NCAs) were proposed as precolumn derivatizing agents for the chiral analysis of asymmetric amines, including α-amino acid alkyl esters [40]. The sample was easily prepared (10 min, room temperature), and the properties of the Fmoc group allowed high sensitivity by fluorescence detection.

A new "active ester-type" (*S*)-*N*-(4-nitrophenoxycarbonyl)-Phe methoxyethyl ester [(*S*)-NIFE] produced by Solvay Peptisyntha was introduced as CDA by Péter et al. [41]. The diastereomeric derivatives produced were separated by reversed-phase liquid chromatography and were detected at 205 nm. The effect of the pH, excess reagent and reaction time on derivatization kinetics, and of the organic modi-

Table 3
Chiral derivatization of amino group of amino acids

Reagent(s)	Enantiomers separated	Detection	References
Reagents based on activated carboxylic acids and their derivatives			
1,3-Benzodioxole-4 or 5-carboxylic acid	Proteinogenic amino acids	Fl, exc: 310 nm; Em:370–380 nm;	[33]
[(*S*)-TBMB-COOH]; [(*S*)-MNB-COOH]	Proteinogenic amino acids	Fl, exc: 310 nm; em: 370–380 nm	[34]; [35–37]
$(Eoc\text{-}Val)_2O$ and $(Eoc\text{-}Phe)_2O$	Ala, Val, Leu, Phe and Ser (Bzl)	UV, 208 nm	[38]
Boc-Phe-NCA, Boc-Asn(Trt)-NCA, Fmoc-Lys(Boc)-NCA, Fmoc-Met-NCA, Fmoc-Ala-NCA, Z-Ala-NCA and Z-Leu-NCA	Proteinogenic amino acids	UV, 254 nm; Fl, exc: 263 nm; em: 313 nm	[39]
N-α-Fmoc-L-Leu-*N*-carboxy anhydrides, *N*-α-Fmoc-L-Ala-*N*-carboxy anhydrides, *N*-α-Fmoc-L-Val-*N*-carboxy anhydrides and *N*-α-Fmoc-L-Phe-*N*-carboxy anhydrides	Proteinogenic amino acids; unnatural amino acids	Fl, exc: 263 nm; em: 313 nm	[40]
(*S*)-NIFE	Proteinogenic amino acids; ring- and α-methyl-substituted phenylalanines	UV, 205 nm	[41]; [42]
(*S*)-NIFE	β-Methyl(alkyl)-substituted phenylalanine, tyrosine, tryptophane and 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid analogs	UV, 205 nm	[43]
(*S*)-NIFE	Proline, pipecolic acid analogs	UV, 205 nm	[44]
(*S*)-NIFE	Piperazine-2-carboxylic acid, morpholine- and thiomorpholine-3-carboxylic acids, analogs with 1,2,3,4-tetrahydroiso-quinoline, 1,2,3,4-tetrahydronorharmane, 1,2,3,4-tetrahydro-2-carboline and 2-benz-azepine skeletons	UV, 205 nm	[45]
(*S*)-NIFE	α-Alkyl- and aryl-substituted prolines	UV, 205 nm	[46]
(*S*)-NIFE	β-3-Homo amino acids	UV, 205 nm	[47]
(*S*)-NIFE	Unusual amino acids	ESI-MS	[57]
Reagents based on chloroformates			
FLEC	α-Hydroxy acids	UV, 265 nm	[49]
FLEC	2-Amino-1,2,3,4-tetrahydronaphthalene-1- and 1,2,3,4-β-carboline-1-carboxylic acids	Fl, exc: 260 nm; em: 315 nm	[50]
FLEC	D,L-Homoalanin-4-yl(methyl)-phosphinate	Fl, exc: 260 nm; em: 305 nm	[51]
NAB-C	β-Adrenoreceptors	Fl, exc: 230 nm; em: 270 nm	[52]
APOC	17 proteinogenic amino acids	UV, 255 nm; Fl, argon laser	[53]
Reagents based on isothiocyanates			
GITC	Metyrosine	UV, 250	[55]
GITC and FDAA	Proteinogenic amino acids, pipecolinic acid in peptides	UV, 254 nm	[56]
GITC and FDAA	β-Methoxytyrosine, *allo*-Thr, *allo*-Ile, *N*-methyl amino acids	ESI-MS	[57]
GITC	Cysteine or cystine in oxytocins	UV, 250 nm	[91]
BGIT		UV, 231 nm	
DDITC, PDITC	19 proteinogenic amino acids	UV, 254 nm	[58,59]
CDITC	Amines	Fl, exc: 333 nm; em:430 nm	[60]
GAL*f*	Ala, Leu; amines	UV, 250 nm	[61]
GATC	β-Blockers	UV, 254 nm	[62]
[(*R*)(−)- and (*S*)(+)-DBD-PynCS] and [(*R*)(−)- and (*S*)(+)-NBD-PynCS]	Val, Phe	Fl, ex: 460–470 nm; em: 530–570 nm	[63]
	14 proteinogenic amino acids		[64]
	17 proteinogenic amino acids		[65]
	D-Amino acids in milk, cream, fermented dairy products, fermented beverage		[66]
	D- and L-amino acids in peptides		[67–71]

Table 3 (*Continued*)

Reagent(s)	Enantiomers separated	Detection	References
DANI	Arg, Asp, Ala, Val, Phe	UV, 245 nm	[74]
	19 proteinogenic amino acids		[75]
	β-Methyl-substituted α-amino acids		[76]
APPI	Val, Phe, Trp salsolinol analogs	UV, 245 nm	[77,78]
Reagents based on *N*-haloarylamino acid derivatives			
FDAA	Neutral, basic, acidic, hydroxy amino acids	UV, 340 nm	[79]
FDAA and GITC	Amino acids with tetrahydroisoquinoline or tetraline ring structures	UV, 340 nm (FDAA) UV, 250 nm (GITC)	[80]
FDAA and GITC	Carboxyglutamic acid analogs	UV, 340 and 250 nm	[81]
FDAA and GITC	β-MePhe, β-MeTyr, β-MeTic	UV, 340 and 250 nm	[82]
FDAA and GITC	β-Methyl amino acids	UV, 340 and 250 nm	[83]
FDAA and GITC	Phe, Tyr, Tic, Trp and tetraline amino carboxylic acid analogs	UV, 340 and 250 nm	[84,85]
FDAA and GITC	β-Alkyl-substituted Trp analogs	UV, 340 and 250 nm	[86]
FDAA and GITC	Ring- and α-methyl-substituted Phe	UV, 340 and 250 nm	[87]
FDAA and GITC	Unusual Gly and Ala analogs	UV, 340 and 250 nm	[89]
FDAA	Protected and uncoded amino acids	UV, 340 nm	[90]
FDAA, FDVA	Cysteine or cystine in oxytocins	UV, 340 nm	[91]
FDAA	Seleno-amino acids	ICP-MS	[92]
FDAA	2-homoarylglycines	UV, 340 nm	[93]
FDAA and GITC	β-Amino acids possessing alicyclic rings	UV, 340 and 250 nm	[94]
FDAA and GITC	β-Amino acids possessing alicyclic rings	UV, 340 and 250 nm	[95]
FDAA and GITC	β-Amino acids possessing alicyclic rings	UV, 340 and 250 nm	[96]
FDAA and GITC	1,1′-Binaphthyl-substituted α-aminoiso-butyric acid; α,α-disubstituted-β-amino acids	UV, 340 and 250 nm	[97,98]
FDAA and GITC	β-Substituted β-alanines	UV, 340 and 250 nm	[99]
FDAA and (*S*)-NIFE	Spin-labeled β-amino acids	UV, 205 nm (NIFE)	[100]
1-fluoro-2,4-dinitrophenyl-5-(*R*,*S*)-phenylethylamine	L-Isoleucinol, D- and L-phenylalaninols	UV, 340 nm	[101]
FDVA	Proteinogenic amino acids	LC–MS	[79]
FDVA, FDPA, FDIA, FDLA	Proteinogenic amino acids	UV	[102]
FDAA and FDLA	Protein amino acids	UV, NMR	[103,104]
FDAA and FDLA	2,4-Diamino-*n*-butyric acid, Lys, Orn	UV, 340 nm; MS	[105]
s-triazine analogs	Proteinogenic amino acids	UV, 254 nm	[106,107]
FDAA	D-Amino acids in Arg-Lys-Lys-Asp-Val-Tyr	UV, 340 nm	[108]
FDAA	Cys in somatostatin analogs	UV, 340 nm	[109]
FDAA	Suc-Tyr-Glu-Pro-Ile-Pro-Glu-Glu-Ala-Cha-Glu-OH	UV, 340 nm	[110]
FDAA	Amino acids in laxaphycins A and B	UV, 340 nm	[111]
FDAA	Gln, Leu, Val, Asp, Ile in cyclic lactonic heptalipopeptide	UV, 340 nm	[112]
FDAA	D enantiomers of Ser, Ala, Pro, Gln, Asn and Phe in eubacteria, archaea and eukaryotes	UV, 340 nm	[113]
FDLA	*N*-methylalanine, homotyrosine in cyanobacterium	MS	[79]
FDAA	Glu-γ-carbonyl-γ-aldehyde, pentahomoserine, Pro in aeruginopeptin	UV, 340 nm; NMR	[104]
FDAA	β-MeAsp, Ala, Leu, Arg, Glu in cyanobacterium	UV, 340 nm	[103,104]
FDAA	Glu, Phe, *allo*-Ile, Pro, and 3-amino-2,5-dihydroxy-8-phenyl-octanoic acid isolated from cyanobacterium *Nostoc* sp. strain	UV, 340 nm	[114]
FDAA	Antibiotic peptide colistin produced by *Bacillus polymyxa colistinus*	MS	[115]
FDLA	Microcystins in extract of algae and cyanobacteria	MS	[116]
FDAA	D-Ile, D-*allo*-Ile, L-Ile, L-*allo*-Ile in depsipeptide from *Aspergillus carneus*	UV, 340 nm	[117]
FDAA	Amino acids in cyclohexapeptide from fungus *Hirsutella kobayasii*	UV, 340 nm	[118]
FDLA	Trp and Met sulfoxide in microcyclamide	UV, 340 nm	[119]

Table 3 (*Continued*)

Reagent(s)	Enantiomers separated	Detection	References
FDAA	D-Ser and L-Ser ratio in normal and Alzheimer human brain	UV, 340 nm	[120]
FDAA	D-Ser in the cerebrum and cerebellum of *bus* mouse	UV, 340 nm	[121]
FDAA	D-Ser in brains of SAMP8 and SAMR1 strains	UV, 340 nm	[122]
FDAA	D- and L-Ser in purified urine sample of mouse	UV, 340 nm	[123]
FDAA	D- and L-phospho-Ser in rat brain	UV, 340 nm	[124]
Reagents based on o-phthalaldehyde + chiral thiol			
OPA + *N*-acetyl-D-penicillamine	Protein kinase C inhibitor (SPC-100270)	UV, 330 nm	[125]
OPA + *N*-isobutyryl-L- and D-Cys	Protein amino acids in grape, juice, beer, wine, dietary whey drink, hard cheese, yeast extract and honey	Fl, exc: 230 nm; Em: 445 nm	[126]
OPA + *N*-isobutyryl-L- and D-Cys	Protein amino acids in bacitracin, gramicidins A and B, polymyxin B, metanicin C, peptide toxin malformin A and the peptide drugs D-Arg-[Hyp^3-Thr$^{5–8}$-D-Phe^7]-bradykinin, β-casomorphin and α_{s1}-exorphin	Fl, exc: 230 nm; Em: 445 nm	[127]
OPA + *N*-isobutyryl-L- and D-Cys	*Allo*-Ile, α-aminobutyric acid, γ-aminobutyric acid and *allo*-Thr in seawater	Fl, exc: 330 nm; em: 445 nm	[128]
OPA + *N*-isobutyryl-L- and D-Cys	D-Glu, D-*erythro*-β-MeAsp, D-Ala, L-Ala and L-Leu content in microcystins and nodularin peptides	Fl, exc: 330 nm; em: 445 nm	[129]
OPA + *N*-isobutyryl-L-Cys	Unusual amino acids	ESI-MS	[57]
OPA + *N*-isobutyryl-L- and D-Cys	D,L-Selenomethionine determination from Antarctic krill	ICP-MS	[130]
OPA + *N*-acetyl-L-Cys	Protein amino acids in blood, urea	Fl, exc: 340 nm; em: 450 nm	[54]
OPA + *N*-acetyl-L-Cys	Proteinogenic and α-dialkylamino acids in geological samples	Fl, exc: 340 nm; em: 450 nm	[131]
OPA + *N*-acetyl-L-Cys	γ-Vinyl-γ-aminobutyric acid in human serum	Fl, exc: 330 nm; em: 450 nm	[132]
OPA + *N*-acetyl-L-Cys	D enantiomers of Ser, Thr, Ala, Tyr, Val, Trp and Leu in milk and oyster	Fl, exc: 344 nm; em: 443 nm	[133]
OPA + *N*-(*tert*-butylthiocarbamoyl)-L-Cys ethyl ester (BTCC)	Asp, Glu, Ser, Ala, Phe in the aspartate racemase assay	Fl, exc: 335 nm; em: 420 nm	[134]
OPA + 1-thio-β-D-glucose tetraacetate	Trp in food proteins	Fl, exc: 325 nm; em: 420 nm	[135]
o-Phenylenediamine + 2-mercaptoethanol	α-Keto acids from D-amino acids of Ala, Leu, Met Phe and Val	Fl, exc: 341 nm; em: 413 nm	[136]

fier on the separation, was investigated and optimized. The applicability of this procedure was demonstrated by the derivatization and separation of proteinogenic amino acids [41], a series of ring- and α-methyl-substituted Phe-s [42], and β-methyl(alkyl)-substituted Phe, Tyr, Trp and 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid analogs [43]. Excellent resolutions were achieved on the application of (*S*)-NIFE for 19 unnatural secondary amino acids, such as Pro, pipecolic acid analogs, piperazine-2-carboxylic acid, morpholine-3-carboxylic acid and thiomorpholine-3-carboxylic acid analogs, and for analogs containing 1,2,3,4-tetrahydroisoquinoline, 1,2,3,4-tetrahydronorharmane, 1,2,3,4-tetrahydro-2-carboline or 2-benzazepine skeletons [44,45]. For the derivatization of highly constrained α-alkyl- and aryl-substituted Pro analogs, (*S*)-NIFE was the best choice, since other derivatizing agents, such as 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate (GITC) and 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (Marfey's reagent) did not result in the formation of derivatives [46]. The diastereomers were analyzed under reversed-phase conditions by means of gradient elution. The efficiencies of derivatization and separation for β-amino acids, including β-3-homo amino acids, were compared for (*S*)-NIFE and (1*S*,2*S*)-1,3-diacetoxy-1-(4-nitrophenyl)-2-propyl isothiocyanate [(*S*,*S*)-DANI] [47]. Although derivatization proceeded with both CDAs, the separation was much better on the application of (*S*)-NIFE.

2.2. Derivatization with chloroformates

In general, the chloroformates developed for the chiral derivatization of amino groups are fluorescent, and in most cases ensure a very low detection level for amino compounds. The most important chloroformate possessing a reactive functional group is 1-(9-fluorenyl)ethyl chloroformate (FLEC) developed

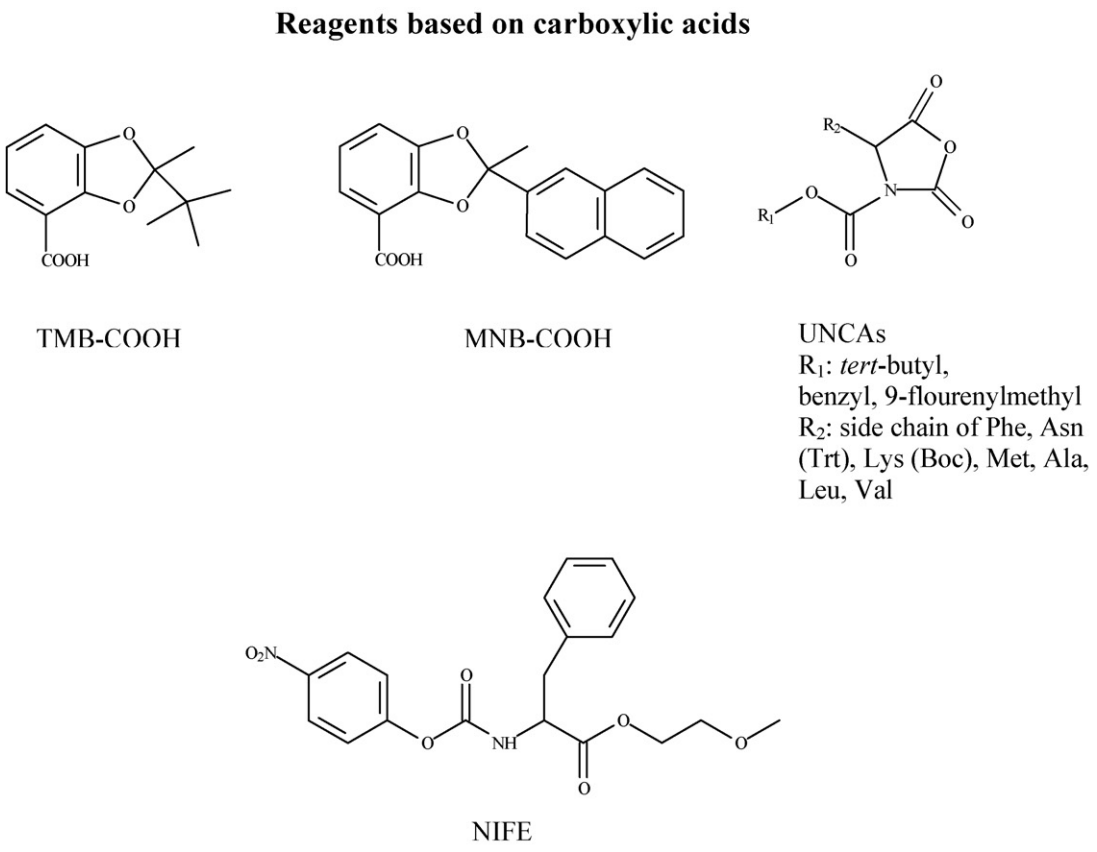

Fig. 1. Chemical structures of derivatizing agents based on carboxylic acids and chloroformates.

by Einarsson et al. [48]. FLEC was initially used for the determination of amino acids by HPLC. The reaction conditions (at room temperature in basic solution) are mild, and the resulting fluorescent diastereomers are stable. Although FLEC equally labels primary and secondary amino acids, for a selective derivatization of secondary amino acids, such as Pro and hydroxy-Pro, the labeling of the amino acids with a primary amino group is carried out with OPA/thiol before the derivatization of the secondary amino acids with FLEC [48]. (+)- and (−)-FLEC were applied as CDAs for the enantioseparation of α-hydroxy acids [49]. Racemates of conformationally constrained 2-amino-1,2,3,4-tetrahydronaphthalene-1-carboxylic acid and 1,2,3,4-β-carboline-1-carboxylic acids were incorporated into oxytocin analogs and, after the chromatographic separation of the diastereomeric peptides, the amino acids in the peptide hydrolyzates were determined by the application of FLEC [50]. The enantiomers of D,L-homoalanine-4-yl(methyl)phosphinate (D,L-GLUF), a nonspecific phosphorus-containing amino acid-

type herbicide, were determined in serum and urine samples by means of FLEC [51]. The derivatization was carried out under mild conditions (40 °C, for 30 min) without racemization induction. New (+)- and (−)-4-(6-methoxy-2-naphthyl)-2-butyl chloroformates (NAB-C) were prepared from the prochiral non-steroidal anti-inflammatory agent nabumetone with the aim of developing an easily detectable chloroformate reagent for the enantiospecific HPLC analysis of amino compounds on achiral stationary phases [52]. The reagent was compared with other reagent containing a 6-methoxy-2-naphthol moiety and was found to be suitable for the analysis of nanogram amounts. New anthracene-based fluorescent reagents, (+)- and (−)-1-(9-anthryl)-2-propyl chloroformates (APOC), were introduced by Thorsen et al. [53], were used for the chiral separation of 17 amino acid enantiomers with a detection limit in the femtomole range. Fig. 1 illustrates the chemical structures of the reagents based on chloroformates.

2.3. Derivatization with isocyanates and isothiocyanates

Derivatization with isocyanate (–NCO)-bearing reagents generally requires more severe reaction conditions than those with isothiocyanate for completion of the derivatization, *i.e.* a longer reaction time and a higher temperature. The applications of these reagents for amino acid analysis were subsidiary in the period in question.

For amino compounds, among the chemically most selective CDAs are isothiocyanates such as 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosil isothiocyanate (GITC), 2,3,4,6-tetra-*O*-benzoyl-β-D-glucopyranosyl isothiocyanate (BGIT) and 2,3,4-tri-*O*-acetyl-α-D-arabinopyranosyl isithiocyanate (AITC) (Fig. 2). Under the reaction conditions applied for the derivatizations, isothiocyanates form the corresponding thiourea derivatives with the primary and secondary amino groups of amino acids.

Kinoshita [54] reviewed the application of GITC in the enantioseparation of proteinogenic amino acids, while the separation abilities of GITC and Marfey's reagent in the resolution of unusual α- and β-amino acids are surveyed in Section 2.4.1.

Hefnawy and James [55] separated metyrosine enantiomers by using GITC. It was found that a reaction time of 2 h at 45 °C resulted in maximum peak heights for metyrosine derivatives. By the application of GITC and Marfey's reagents as CDAs, Bringmann et al. [56] identified the configurations of all proteinogenic amino acids and unusual L-pipecolinic acid in the novel cyclodepsipeptides, petrosifungins A and B. Hess et al. [57] separated β-methoxy-Tyr, *allo*-Thr and *allo*-Ile by the use of different CDAs, such as GITC, (*S*)-NIFE, FDAA and OPA + isobutyryl-L-Cys. Due to the complex relationship between the investigated CDAs, stereochemical analyses with a combination of two or more of the CDAs yielded the most reliable results for a given separation problem.

Three new isothiocyanate-based chiral *trans*-1,2-diaminocyclohexanes, *i.e.* *N*-3,5-dinitrobenzoyl-*trans*-1,2-diaminocyclohexane isothiocyanate [(*S*,*S*)- and (*R*,*R*)-DDITC] [58], *N*-[(2-isothiocyanato)cyclohexyl]pivalinoyl amide (PDITC) [59] and *N*-[(2-isothiocyanato)cyclohexyl]-6-methoxy-4-quinolinylcarboxamide (CDITC) [60], were synthesized (Fig. 2). The derivatives obtained with DDITC, PDITC, and CDITC are well separated by HPLC. The separation factors and resolutions of the diastereomeric thioureas were higher than those of the GITC derivatives, which are well established as the labels for primary and secondary amines. Although DDITC and PDITC have been developed as UV labels, the derivative obtained from CDITC fluoresces highly at 430 nm (excitation at 333 nm). Nineteen proteinogenic amino acids were labeled with CDITC and separated by reversed-phase HPLC and CE [60]. The separation and reproducibility with HPLC were superior to those with CE. The above three CDAs, which exhibit higher separation efficiency than GITC, are good labels for primary and secondary amines. However, the derivatization conditions for GITC are milder than those for these reagents (60 °C and 2 h in the presence of sodium carbonate).

New CDAs with sugar moieties, enantiomerically pure 2,3,4,6-tetra-*O*-benzoyl-β-D-galactofuranosyl isothiocyanate (GAL*f*) [61] and 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl isothiocyanate (GATC) [62] were designed for the enantioseparation of amines, including some proteinogenic amino acids. The resolution was readily accomplished on both analytical and preparative scales.

Toyo'oka and coworkers developed fluorescent chiral tagging reagents with the benzofurazan (2,1,3-benzoxadiazole) structure for the enantioseparation of various types of racemates involving amino, carboxyl, carbonyl and hydroxy functional groups. For derivatization of the amino group of amino acids, optically active fluorescent Edman-type reagents bearing a –NCS functional group were synthetized, *i.e.* *R*(−)- and *S*(+)-4-(3-isothiocyanatopyrrolidin-1-yl)-7-(*N*,*N*-dimethylaminosulfonyl)-2,1,3-benzoxadiazole [(*R*)(−)- and (*S*)(+)-DBD-PynCS], and *R*(−)- and *S*(+)-4-(3-isothiocyanatopyrrolidin-1-yl)-7-nitro-2,1,3-benzoxadiazole [(*R*)(−)- and (*S*)(+)-NBD-PynCS] [63] (Fig. 2). The reactions of amino acids with these reagents at 55 °C proceeded within 20 min in the presence of a basic catalyst. The fluorescence properties of the derivatives obtained from the DBD and NBD moieties are preferred for the analysis of real samples because of their long wavelength (excitation at 460–470 nm, and emission at 530–570 nm) and high fluorescence intensities. The thiocarbamoyl derivatives of proteinogenic amino acids for neutral and aromatic amino acids were better resolved than those of basic and acidic amino acids [64]; the separation of 17 D,L-amino acids proved difficult with a single chromatographic run, even when gradient elutions were adopted [65]. The D-amino acid contents were determined in milk, cream, fermented dairy products, fermented beverages and human urine, and the D-amino acids were identified by using both isomers of DBD-PyNCS and by online HPLC-ESI-MS [66]. These chiral reagents were applied for determination of the stereochemical purities of peptides, such as D,L-Ala and D,L-Leu-containing di- and tripeptides [67]; neurotensin, [D-Ala^2,D-Leu^5]enkephalin and morphine tolerance peptide [68]; [D-Ala^2]-Leu-enkephalin [69]; [D-Ala^2]-Leu-enkephalin, [D-Ala^2]deltorphin II, D-Phe-Met-Arg-Phe-amide, Phe-D-Met-Arg-Phe-amide, β-lipotropin, Asp-Ser-Asp-Pro-Arg and

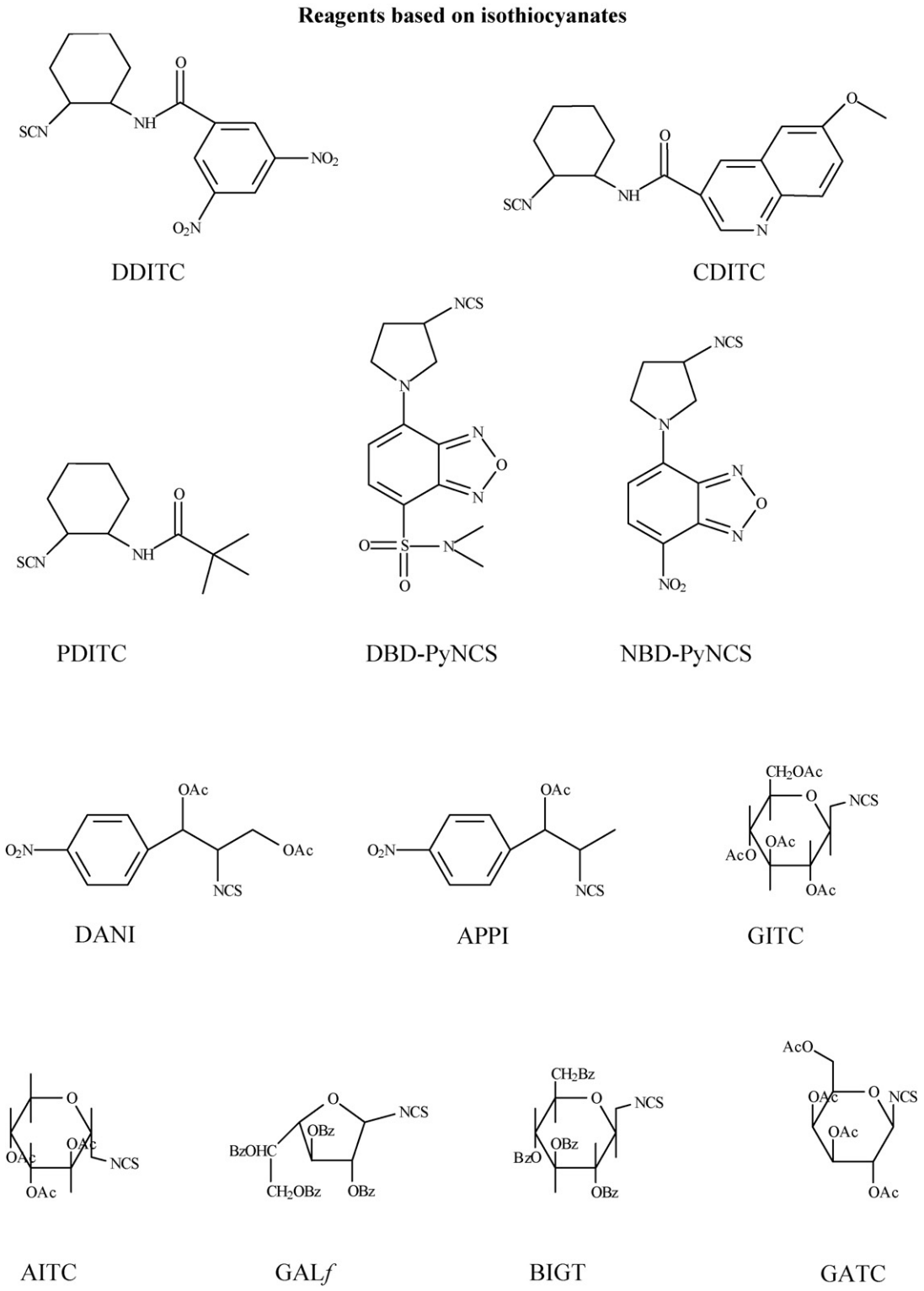

Fig. 2. Chemical structures of derivatizing agents based on isothiocyanates.

Pro-Asp-Val-Asp-His-Val-Phe-Leu-Arg-Phe amide [70]; and [L-Ala^2]-Leu-enkephalin and [D-Ala^2]-Leu-enkephalin [71]. The results of the application of DBD-PyncS and NBD-PyncS CDAs are reviewed in the papers of Toyo'oka [28,72,73].

New CDAs, readily available in both enantiomeric forms, (1*S*,2*S*) or (1*R*,2*R*)-1,3-diacetoxy-1-(4-nitrophenyl)-2-propyl isothiocyanate (DANI), were synthetized by Péter et al. [74], as was (1*S*,2*R*)-1-acetoxy-1-phenyl-2-propyl isothiocyanate (APPI) by Péter et al. [77]. Their applicability was demonstrated on the examples of the resolution of a series of proteinogenic α-amino acids [75], β-methyl-substituted α-amino acids, β-amino acids with cycloalkane skeletons [76] and 17 unnatural β-amino

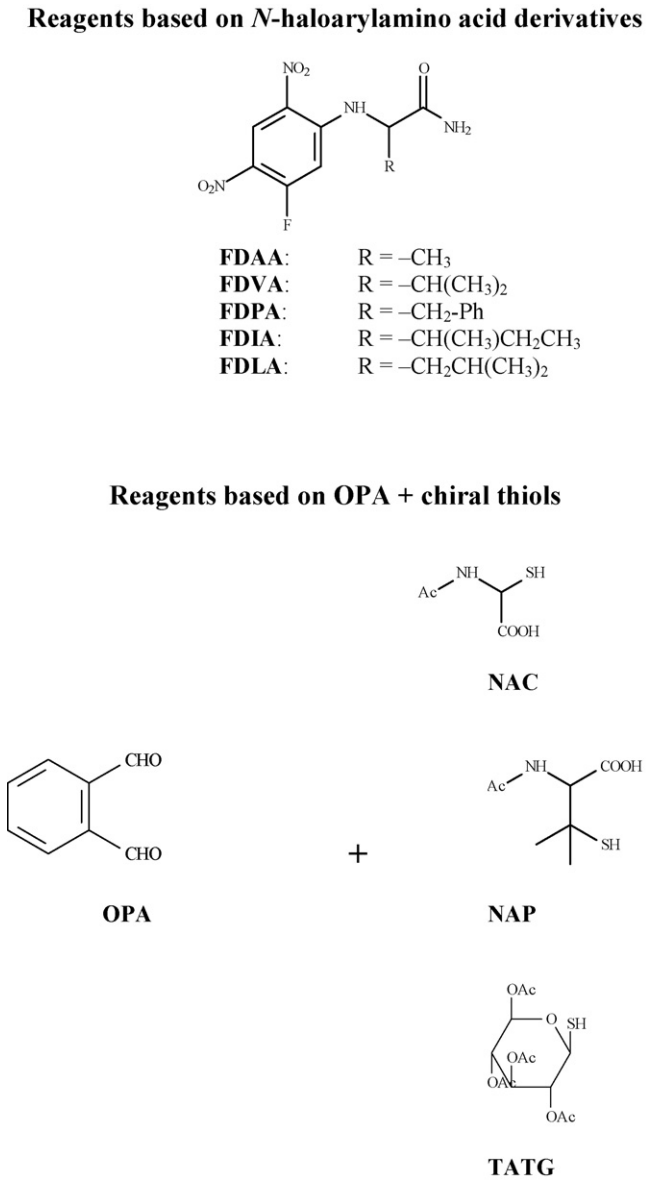

Fig. 3. Chemical structures of derivatizing agents based on *N*-haloarylamino acid derivatives and OPA + chiral thiols.

acids, including β-3-homo amino acids [47]. It was also demonstrated that, in the enantioseparation of Val, Phe, Trp [77] and salsolinol analogs, the application of APPI was as favorable as that of DANI [78]. For the chemical structures of reagents based on isothiocyanates, see Fig. 2.

2.4. Derivatization with N-haloarylamino acid derivatives

Marfey's reagent, 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide, (FDNP-Ala-NH_2 or FDAA), a chiral variant of the Sanger's reagent (2,4-DNFB), is one of the most widely used reagents for the resolution of amino acid enantiomers. (The chemical structures are presented in Fig. 3.) The nucleophilic attack of the α- or β-amino group of the amino acids on the C–F bond activated by the two nitro groups on the aromatic ring results in a smooth reaction to yield diastereomeric aniline derivatives with good UV detectability. The most frequently reported use for Marfey's reagent has been in the synthesis of amino acid analogs and synthetic peptides. B'Hymer et al. [29] and Bhushan and Brückner [30] have produced excellent background reviews on the application of Marfey's reagent.

2.4.1. Applications and comparison with other derivatizing reagents

The applicability and limitations of Marfey's method were extensively examined by Harada et al. [79]. The neutral amino acids displayed good resolution between the L and D diastereomers, where as the separation for hydroxy and acidic amino acid derivatives was very poor. Basic amino acids gave three derivatives and indicated that an α-amino group is essential for the resolution of both diastereomers. For standard α-amino acids, except for a few basic amino acids, the Marfey's method proved to have wide applicability.

Optical isomers of conformationally constrained amino acids with tetrahydroisoquinoline or tetraline ring structures [80] and of γ,γ′-di-*tert*-butyl-D,L-γ-carboxyglutamic acid and D,L-γ-carboxyglutamic acid [81] were synthetized, characterized and identified by the application of precolumn derivatization with FDAA and GITC. In general, FDAA was much favorable as a derivatizing agent than GITC, giving better peak shapes and higher resolution. Relating the elution sequence to the configuration of the amino acids does not seem to be warranted since reversal of the elution sequence occurred. Four enantiomers of β-MePhe, β-MeTyr and 1,2,3,4-tetrahydroisoquinoline-β-methyl-3-carboxylic acid (β-MeTic) were derivatized with FDAA and GITC and were separated by HPLC on a C18 column. The best resolution of the four enantiomers in all cases was achieved for the FDAA derivatives [82,83]. New conformationally constrained unusual amino acid analogs of Phe, Tyr, Tic, Trp and tetraline amino carboxylic acids were synthetized and the enantiomers were separated by an indirect method with the application of FDAA or GITC or with chiral columns [84–86]. Good separations were achieved with FDAA or GITC in most cases, while for β-alkyl-substituted Trp analogs GITC sometimes displayed better resolution for the four stereoisomers [86]. The enantiomers of ring- and α-methyl-substituted Phe and Phe amides were resolved by derivatization with FDAA and GITC or with chiral columns [87]. Application of the original protocol of Marfey [88] proved unsuccessful. In the modified procedure, higher reactant concentrations and longer reaction times were used. The use of a higher temperature and a longer reaction time may promote the racemization of enantiomers containing hydrogen in the α position. Of the two indirect methods, GITC derivatization was more effective than FDAA derivatization. Unusual Gly and Ala analogs were synthetized and the enantiomers were separated by indirect and direct methods [89]. In the indirect method, FDAA seemed more efficient than GITC for the separation of Gly and Ala analogs. The FDAA derivatives generally exhibited a 40–60% higher selectivity factor, which resulted in much higher resolution.

Szabó et al. [90] developed enantiomeric derivatization of uncoded amino acids and performed comparative racemization studies, using Marfey's method. For separation of epimeric

mixtures of oxytocin and other peptides containing cysteine or cystine various CDAs were used including OPA with *N*-isobutyryl-L-cysteine, GITC, BGIT, FDAA and FDVA [91]. FDAA was suitable for the separation of homocysteic acid, while FDVA was suitable for the separation of L- and D-cysteic acid. FDVA provides longer retention times than FDAA for the determination of configuration and stereochemical purity of the Cys residues in peptides. Montes-Bayon et al. [92] successfully used Marfey's reagent for the chiral purity analysis of seleno amino acids in selenium-inoculated yeast and synthetic selenomethionine in pharmaceutical preparations. The enantiomeric excess of 2-homoarylglycines, during their asymmetric syntheses, was determined by NMR analysis after derivatization with Marfey's reagent [93].

β-Amino acids possessing alicyclic rings derivatized with FDAA and GITC have been successfully separated and identified by HPLC analysis. The four stereoisomers of *cis*- and *trans*-2-amino-cyclopentane-1-carboxylic acid [94], and *cis*- and *trans*-2-amino-cyclohexene-1-carboxylic acid [95] have been successfully separated as FDAA and GITC derivatives, but the separation of *cis*-(1*S*,2*R*)- and *trans*-(1*R*,2*R*) stereoisomers as FDAA derivatives was not satisfactory. Stereoisomers of β-amino acids possessing bicyclo[2,2,1]heptane rings [96], such as *diendo*- and *diexo*-3-aminobicyclo[2,2,1]heptane-2-carboxylic acids and *diendo*- and *diexo*-3-aminobicyclo[2,2,1]heptene-2-carboxylic acids were more favorably separated as the FDAA derivatives than as the GITC derivatives. It is interesting that enantiomers of molecules of α-amino acids with axial chirality, such as free and *N*-protected (*R*,*S*)-2′,1′:1,2;1″,2″:3,4-dinaphthcyclohepta-1,3-diene-6-amino-6-carboxylic acid (Bin) were nicely separated as the GITC derivatives, while the FDAA derivatives displayed very poor resolution [97]. On the other hand, the β-amino acid version of such a molecule, (*R*,*S*)-2′,1′:1,2;1″,2″:3,4-dinaphthcyclohepta-1,3-diene-6-aminomethyl-6-carboxylic acid (β^2-Bin), and the N- and/or C-terminal-protected derivatives underwent very nice separation as the FDAA derivatives, whereas the GITC derivatives were not resolved [98]. Similarly, FDAA has been found to be more efficient in comparison with GITC as the CDA for the HPLC resolution of several β-substituted β-alanines: the resolution was 2–5 times better for the FDAA derivatives than for the GITC derivatives [99]. Steroisomers of spin-labeled β-amino acids were separated by direct and indirect methods [100]. The indirect separation through the use of FDAA failed because of the low yield of the derivatization reaction. Separation was successful when GITC or (*S*)-*N*-(4-nitrophenoxycarbonyl-Phe methoxyethyl ester [(*S*)-NIFE] was applied as CDA.

2.4.2. Chiral variants of 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide

In order to evaluate the effects of substituents in Marfey's reagent, new reagents in which Ala was replaced by other chiral constituents were synthetized and applied. Harada et al. [101] prepared a chiral anisotropic reagent, 1-fluoro-2,4-dinitrophenyl-5-(*R*,*S*)-phenylethylamine, for determination of the absolute configurations of the α-carbon in primary amino compounds. The amino compounds analyzed included L-isoleucinol and D- and L-phenylalaninols.

New FDAA analogs were applied in combination with ESI-MS by means of frit-fast atom bombardment [79,102,103]. With the Marfey [88] procedure Fujii et al. [102] prepared Marfey's analogs 1-fluoro-2,4-dinitrophenyl-5)-L-Val amide (FDVA), -L-Phe amide (FDPA), -L-isoLeu amide (FDIA), -L-Leu amide (FDLA) and -D-Ala amide (FDAA), in which the L-Ala amide was replaced by each amino acid amide, and this was followed by analyses of the protein and non-protein amino acid derivatives by LC/MS. (The chemical structures of these reagents are illustrated in Fig. 3.) Fujii et al. [103,104] replaced FDAA by FDLA, and observed that this provided enhanced sensitivity, hydrophobicity and thermal stability in comparison with L-FDAA. The amino acids derivatized with FDLA displayed almost the same retention behavior as that with FDAA. Fujii et al. [102] investigated the separation mechanism and determined the absolute configurations by using UV and NMR techniques sensitive to conformation. The main conclusion was that the FDAA derivatives of D-amino acids assume a *cis* arrangement because the side-chain in most of the α-amino acids is more hydrophobic than the carboxylic group. Accordingly the FDAA derivative of the L enantiomer is usually eluted before the corresponding D isomer. Participation of the nitro groups in hydrogen bonding is supported by the fact that *s*-triazine CDAs, which are considered to be structurally related to FDAA, are in general less effective for the indirect chiral separation of amino acids [106].

Harada et al. [105] observed the abnormal elution behavior of ornithine (Orn) derivatized with *bis*-1-fluoro-2,4-dinitrophenyl-5-L-Leu amide and *bis*-1-fluoro-2,4-dinitrophenyl-5-L-Ala amide: the sequence of elution of Orn was opposite (D < L) in spite of the relatively long retention time, while the L isomers of the *bis*-1-fluoro-2,4-dinitrophenyl-5-L-Ala amide of 2,4-diamino-*n*-butanoic acid and Lys eluted before the corresponding D isomer in HPLC. The NMR and UV spectral analysis did not provide any structural information. Hence, despite the low resolution power, use of the 2,4-dinitrophenyl-5-phenylethylamine derivative was recommended for the analysis of Orn instead of *bis*-1-fluoro-2,4-dinitrophenyl-5-L-Leu amide derivative.

From considerations of structural analogy and reactivity, Brückner and Wachsmann [106] replaced the dinitrofluoro moiety of FDAA by a *s*-triazine moiety having a suitable chromophore. These chiral monohalo-*s*-triazines were found to be capable of resolving certain D,L-amino acids. The resolution was lower in some cases in comparison with those obtained on the reaction of the same D,L-amino acids with other chiral variants [107].

Marfey's reagent is frequently used for the chiral purity determination of amino acids in peptide (Table 3). After the acid hydrolysis of peptides the use of Marfey's reagent has been demonstrated to generate accurate results with little racemization of amino acid isomer residues [108]. The chiral composition of Cys in somatostatin analogs was determined by Jacobson et al. [109]. The synthetic decapeptide Suc-Tyr-Glu-Pro-Ile-Pro-Glu-Glu-Ala-Cha-Glu-OH was hydrolyzed and analyzed for chiral

purity by B'Hymer [110] and excellent chromatographic resolution of the five pairs of natural amino acids was obtained. The configurations of the amino acids of laxaphycins A and B (cyclopeptides), produced by the tropical marine cyanobacterium *Lyngbya majuscule*, were established by HPLC analysis [111]. Mikkola et al. [112] analyzed D- and L-Gln, Leu, Val, Asp and Ile in cyclic lactonic heptalipopeptides isolated from three *Bacillus licheniformis* strains. The D-enantiomers of Ser, Ala, Pro, Gln, Asn and Phe were determined in various eubacteria, archaea and eukaryotes, such as *Staphylococcus epidermidis*, *Streptococcus pyogenes*, *Thiobacillus ferrooxidans*, *Pyrobaculum islandicum* [113]. FDLA with MS detection was used to determine the absolute configuration of *N*-methyl-Ala and homo-Tyr produced by the cyanobacterium *Anabaena flos-aquae* [79], for determination of Glu-γ-carbonyl-γ-aldehyde, pentahomo-Ser and Pro in aeruginopeptin 228A [104], and for chiral analysis of β-MeAsp, Ala, Leu, Arg and Glu produced by the cyanobacterium *Microcystis aeruginosa M228* [103,104]. Marfey's method was used to determine the absolute configurations of Glu, Phe, *allo*-Ile, Pro, and (2*S*,3*R*,5*R*)-3-amino-2,5-dihydroxy-8-phenyloctanoic acid isolated from the cyanobacterium *Nostoc* sp. strain 152 [114]. The antibiotic peptide colistin produced by *Bacillus polymyxa colistinus* and microcystins in extracts of various algae and cyanobacteria were analyzed by means of derivatization of FDAA and FDLA by application of an HPLC/MS technique [115,116]. By applying Marfey's method, Capon et al. [117] differentiated D-Ile from D-*allo*-Ile, or L-Ile from L-*allo*-Ile in the hydrolyzate of the depsipeptide isolated from *Aspergillus carneus*, while differentiation of L-Ile from L-*allo*-Ile in a new cyclohexapeptide isolated from the fungus *Hirsutella kobayasii* BCC 1660 was achieved with a chiral column [118]. After flash hydrolysis the absolute configuration of thiazole-containing amino acids in microcyclamide was determined by using FDLA for derivatization [119].

HPLC after derivatization with FDAA has been routinely applied for the determination of D-Ser in the cerebrum and cerebellum of *bus* mouse [121] and in the brains of the SAMP8 and SAMR1 strains [122], and for determination of the D-Ser and L-Ser ratio in normal and Alzheimer human brains [120]. Analysis of the Ser isomer composition in purified urine samples from mice [123] and the separation and quantitation of D- and L-phosphoserine in rat brain [124] were carried out by FDAA derivatization followed by HPLC.

2.5. Application of OPA + chiral thiol reagents

Another general reaction for the chiral derivatization of amino acids is their reaction with OPA and chiral thiols to form fluorometrically highly active isoindole derivatives. The OPA + chiral thiol system is widely applied for determination of the amino acid enantiomer contents in living systems, food analyses and geological systems, with fluorescence detection (Table 3). Rajewski et al. [125] used OPA and *N*-acetyl-D-penicillamine (NAP) for the derivatizaton of a protein kinase C inhibitor (SPC-100270). The reaction was complete within 3 h at 50 °C, and the separation of isoindole derivatives was achieved with isocratic elution. The D-amino acid contents in grape juice, beer, wine, dietary whey drink, hard cheese, yeast extract and honey [39]) and in peptide antibiotics such as bacitracin, gramicidins A and B, polymyxin B, metanicin C, peptide toxin malformin A and the peptide drugs D-Arg-[Hyp^3-Thi$^{5-8}$-D-Phe^7]-bradykinin, β-casomorphin and α_{s1}-exorphin [126] were analyzed through the application of OPA and *N*-isobutyryl-L-Cys or *N*-isobutyryl-D-Cys as derivatizing agents. For the detection of the isoindoles, their fluorescence at 445 nm when excited at 230 nm was utilized, aqueous acetate buffer–methanol–acetonitrile being used as mobile phase. The results provide further proof that D-amino acids are common in microbially fermented foods and that microorganisms (bacteria and yeasts) are major, but not exclusive, sources of free D-amino acids occurring in foods. In peptides, low amounts of D enantiomers (0.1–0.9%) were detected. Further, OPA and *N*-isobutyryl-L-Cys or *N*-isobutyryl-D-Cys derivatization was used for the enantiomeric determination of *allo*-Ile, α-aminobutanoic acid, γ-aminobutanoic acid and *allo*-Thr in seawater and fossil samples [128], for determination of the D-Glu, D-*erythro*-β-MeAsp, D-Ala, L-Ala and L-Leu contents in microcystins and nodularin peptides [129] and for D,L-selenomethionine determination in Antarctic krill [130]. An OPA and *N*-acetyl-L-Cys derivatization system was applied for the enantiomeric determination of proteinogenic and α-dialkylamino acids in geological samples [131] and of vigabatrin enantiomers (γ-vinyl-γ-aminobutanoic acid) in human serum [132], and for determination of the D enantiomers of Ser, Thr, Ala, Tyr, Val, Trp and Leu in milk and oyster samples [133]. A novel chiral thiol reagent, *N*-(*tert*-butylthiocarbamoyl)-L-cysteine ethyl ester (BTCC), was developed by Nimura et al. [134] and applied together with OPA for enantiomer determination of the amino acids Asp, Glu, Ser, Ala and Phe in the aspartate racemase assay. Fluorescence detection was carried out with excitation at 335 nm and emission at 420 nm. Racemization of Trp in the course of hydrolysis of food proteins in the presence of *p*-toluenesulfonic acid was followed by derivatization with OPA and 1-thio-β-D-glucose tetraacetate [135]. The method was suitable to follow the racemization; other reactions, *e.g.* oxidative deterioration in the Trp content, were also observed. A new strategy for the selective determination of D-amino acids was developed by Oguri et al. [136]. D-Amino acids were enzymatically modified with D-amino acid oxidase. The α-keto acids formed were derivatized with *o*-phenylenediamine in the presence of 2-mercaptoethanol to give fluorescent quinoxalinol derivatives. The D enantiomers of Ala, Leu, Met Phe and Val in *Ruditapes philippinarum* were analyzed after enzymatic digestion with D-amino acid oxidase and fluorescence detection (excitation at 341 nm, and emission at 413 nm).

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