

# Fluorescent Chiral Derivatization Reagents Possessing Benzofurazan Structure for the Resolution of Optical Isomers in HPLC: The Synthesis, Characteristics and Application

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**Abstract:** Indirect resolution of chiral molecules, based upon pre-column derivatization and diastereomer formation using benzofurazan bearing chiral labeling reagents, by high-performance liquid chromatography are described in this mini-review. The synthesis, characteristics and application of the fluorescent chiral derivatization reagents for various functional groups, i.e. amine (NBD-PyNCS, DBD-PyNCS, DBD-Pro, DBD-hydroxyproline), carboxyl (NBD-APy, DBD-APy, ABD-APy), carbonyl (NBD-ProCZ, DBD-ProCZ), hydroxyl (NBD-Pro-COCl, DBD-Pro-COCl) and thiol, etc., are including in the text.

**Keywords:** Benzofurazan fluorophore, chiral resolution, biological important molecule, diastereomer formation, HPLC analysis.

## INTRODUCTION

Many chiral compounds have been determined with the direct resolution that employ CSP columns containing immobilized chiral selector [1]. The separation mechanism is owing to the stability difference of the diastereomeric complexes formed between CSP and each enantiomer in the flow system. Since the method requires no pre-treatment such as derivatization, possible racemization during the separation seems to be negligible. The direct resolution using CSP column is thus preferable for trace analysis of antipode enantiomer in main component. The separation is highly influenced on the interaction between CSP and enantiomer. Therefore, the choice of the best column for separation of each enantiomer is fairly difficult. The elution order of a pair of enantiomers is also dependent upon the CSP column used, and can not be changed easily. Furthermore, the detection sensitivity is sometimes insufficient in real sample analysis because the sensitivity is dependent upon the racemate itself.

The indirect resolution involving a derivatization step with a chiral tagging reagent is an efficient technique for separation of many racemates [2-6]. The resolution is based upon diastereomer formation by the reaction with a chiral derivatization reagent. A pair of enantiomers is labeled with a chiral derivatization reagent to generate corresponding to a couple of diastereomers. The separation is performed with the differences from physicochemical properties (e.g., stereochemistry and stability) with achiral stationary phase. The elution order and degree of separation of the diastereomer derived from each reagent can not easily predict with conventional achiral stationary phase column such as ODS. As the separation is influenced with the

distance between two asymmetric centers of substrate and the reagent, the distance should be minimize to get good separation. The conformational rigidity around the chiral centers is another important factor for the separation [7]. Thus, it is recommended a resolving reagent that free rotation near the asymmetric center in the substrate is interfered by the formation of the diastereomer. However, there is no obvious rule concerning the separation of both diastereomers. The good sensitivity and selectivity of indirect method coupled with an efficient detection system are attractive means for the determination of chiral molecules in real sample analysis.

Since the optical purity of chiral derivatization reagents is generally less than 99.5%, strict assay of trace quantity of enantiomer in large amount of antipode is relatively difficult in indirect method. Thus, the method is recommended for the analysis, such as metabolic study in biological specimens, because the % CV is usually in the acceptable range of error. Therefore, this indirect derivatization method is suitable for trace analysis of enantiomers in biological samples, such as blood and urine, because highly sensitive detection is performed with option of coupling with suitable reagents which have high molar absorptivity ( ) or high fluorescence quantum yield ( ).

The selection of the reagent dominates accuracy, precision and repeatability of the quantitative analysis. Several important points worthy of consideration for the choice of chiral derivatization reagent are as follows: (i) Optical purity of the reagent should be as great as same as the chemical purity. Since the opposite enantiomer contaminating the reagent also produces a corresponding diastereomer, it is obvious that erroneous results will be obtained with the use of impure reagents. (ii) The degree of racemization during labeling reaction and storage of the reagent itself is another important issue for quantitative determinations. Furthermore, the chemical stability of the resulting diastereomers also influences the results. Good

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stability at least one day is required, because autoanalysis overnight is usually planned for many samples. (iii) The reactivity of the reagent for each enantiomer and the FL properties (wavelength and intensity) of the resulting derivatives are essentially the same. When the reaction rates are extremely different in both enantiomers, the reaction condition should be optimized carefully. In the case of difference of FL properties, each calibration curve should be drawn for the determination of a pair of enantiomers. (iv) The reagent possess specificity for the target functional group and quantitatively labels the analyte under mild conditions. (v) The resulting diastereomers exhibit an adequate detector response for sample analysis. (vi) Another important point is the solubility of the reagent whether it is freely soluble in water or miscible in aqueous solvents, such as methanol and acetonitrile, because many bioactive chiral molecules exist in aqueous solution. (vii) It is another importance with practical mean that both enantiomers of the reagent are commercially available or easily obtained by simple synthesis. Because the elution order can be controlled with the selection of the reagent enantiomer. This is necessary when the determination of a trace enantiomer is required in the presence of a large amount of antipode enantiomer. These items listed here are general importance for all chiral tagging reagents, not only for FL but also UV-VIS.

Labeling of chiral compounds with the reagent that afford the structures absorbing UV or VIS region is the most popular means of derivatization. There are various chiral derivatization reagents for HPLC that provide UV-VIS absorption. It is predominant that the derivatives obtained from the reagents have strong absorption in long wavelength region, because many endogenous substances, which absorb in relatively short wavelengths, are shown in real samples. Since the interference of impurities absorbing the detection wavelength is considered in real samples, especially in complex matrices such as biological specimens, the reagents absorbed in VIS band are preferable in terms of selectivity. Although a number of UV labels have been applied to the tagging of various functional groups, the sensitivity of the derivatives is not good enough in some real samples.

To solve the disadvantage, various types of FL labels have been developed and many papers concerning FL tagging have been published. The FL label is the most effective for determinations in biological specimens, in terms of sensitivity and/or selectivity. Different types of FL labeling reagents have been developed for the enantioseparation of biologically important molecules. The labeling with chiral derivatization reagent is carried out with the reaction of a reactive functional group in substrates, e.g., amines (primary and secondary), carboxyl, carbonyl, hydroxyls (alcohol and phenol) and thiol. Many organic reactions are adopted for the labeling of various functional groups in target molecules.

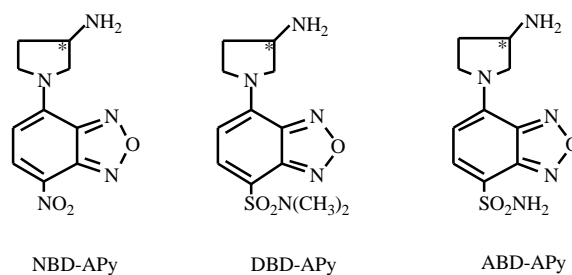
A number of optically active reagents have been developed for HPLC analysis of chiral molecules having various functional groups [2-6]. Among the functional groups, the tagging reactions of primary and secondary amines have been extensively investigated, because chiral primary and secondary amines are easy to label with carboxyls, chloroformates, isocyanates and isothiocyanates

to yield corresponding amides, carbamates, ureas and thioureas, respectively. Racemic carboxylic acids are usually labeled with chiral primary amine in the presence of activation reagents such as EDC. The reaction proceeds under mild condition at room temperature. The ester formation with chiral alcohol is also adopted for the derivatization of carboxyls. Since the reaction conditions are generally drastic, possible racemization should be monitored during the reaction. The labeling of alcoholic hydroxyl group is most difficult, because the reaction competes to water in the medium. Hence, the reaction with acid chloride type reagents is mainly performed in anhydrous solvents such as chloroform and benzene. Variety of organic reactions is possible for the carbonyl compounds, aldehydes and ketones. However, few labels are reported for the resolution of carbonyl enantiomers. Those are amine and hydrazine type reagents to produce corresponding nitriles and hydrazones.

Although various FL labeling reagents have been developed for the resolution of chiral molecules, this review focused on benzofurazan type reagents. The synthesis, characteristics and application of chiral derivatization reagents having benzofurazan structure for the resolution of optical isomers by HPLC are described. The reagents include DBD-APy, NBD-APy, ABD-APy, DBD-PyNCS, NBD-PyNCS, DBD-Pro-COCl, NBD-Pro-COCl, DBD-ProCZ, NBD-ProCZ, DBD-Pro, DBD- -Pro, and DBD-OH-Pro, etc.

## REAGENTS FOR CARBOXYLIC ACID

*R*- and *S*-enantiomer of DBD-APy, NBD-APy or ABD-APy have been developed for the resolution of carboxylic acid enantiomers in HPLC (Fig. 1) [8]. The chiral derivatization reagents were quantitatively synthesized from one-step reaction of optical active APy with corresponding 4-fluoro-2,1,3-benzoxadiazoles (DBD-F, NBD-F and ABD-F). The reactions proceed under mild condition at room temperature, and thus the racemization during the reaction was negligible. The optical purity of the synthesized reagents, which were determined from a CSP column (ULTRON ES-PhCD), was higher than 99.5 % [9].



**Fig. (1).** Structures of fluorescent chiral derivatization reagents for carboxylic acids.

Carboxylic acids such as *N*-acetylamino acids and anti-inflammatory drugs (e.g. naproxen and ibuprofen) react with DBD-APy (*S*(+)- and *R*(-)) or NBD-APy (*S*(+)- and *R*(-)) at room temperature in the presence of DPDS and TPP as the activation agents [9]. Although the reaction time to obtain quantitative yield is different in each carboxylic acid, 1hr reaction at room temperature is usually enough. The reaction

time could not be reduced with heating. The chiral carboxylic acid was converted to the corresponding amide diastereomer. The reactivities of *R*- and *S*-DBD-APys to each enantiomer of carboxylic acid (naproxen) are comparable. However, slight difference of the FL intensities was observed in the resulting diastereomers. The derivative obtained from *D*-naproxen and *S*-DBD-APy was the highest. The maximal fluorescence excitation and emission wavelengths were around 470 nm and 580 nm (DBD-APy derivative), 470 nm and 550 nm (NBD-APy derivative), and 470 nm and 580 nm (ABD-APy derivative). The fluorescence intensities were stable at pH 2-8, and highest intensities were obtained from DBD-APy derivatives. The emission maxima shifted to shorter wavelength region together with increase of organic solvent in the medium. The fluorescence intensities also elevated with the increment.

The resulting diastereomers derived from DBD-APy were completely separated by both reversed-phase and normal-phase chromatography [9]. The *R<sub>s</sub>* values in reversed-phase and normal-phase chromatography were 1.62-6.96 and 2.58-7.60, respectively. Although the complete resolutions of the enantiomers of carboxylic acids were achieved by the normal-phase chromatography, the method is not recommended because biologically important chiral molecules generally exist in aqueous matrix. The elution order of the derivatives was possible to change using the opposite enantiomer of the reagents. The detection limits of chiral carboxylic acids was 10-30 fmol levels with conventional fluorescence detector.

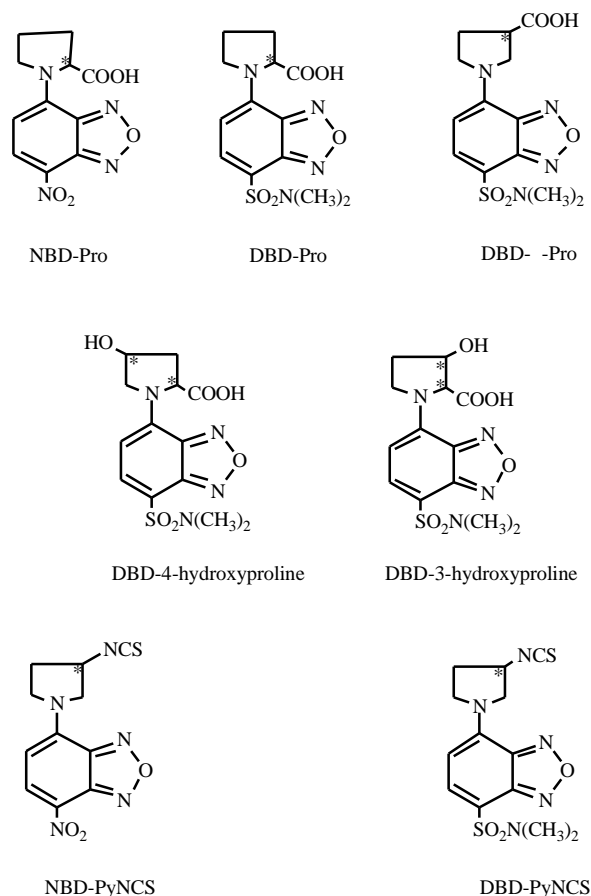
The excitation wavelength of the NBD-APy derivative was around 480nm and thus matched for the wavelength of argon-ion laser. Thus, the diastereomers derived from NBD-APy provided sensitive detection not only with conventional fluorescence detection but also with argon-ion (488nm) LIF detection [9,10]. The sensitivity was depending upon the laser source used. Although higher laser power produced higher peaks, the baseline noises also increased. Consequently, the detection sensitivity has to be considered the signal-to-noise ratio. The detection limits of naproxen derivatives of DBD-APy, NBD-APy and ABD-APy on reversed-phase chromatography were 10, 5 and 30 fmol, respectively. Furthermore, the use of semi-micro column instead of conventional column increased the sensitivity to 1 fmol level.

On the other hand, the DBD-APy derivatives were also amenable to the peroxyoxalate CL [11]. Oxalate ester and H<sub>2</sub>O<sub>2</sub> were employed for the excitation of the carboxylic acid (DL-naproxen) derivatives. The CL reaction depends upon reaction pH. The optimum pH was different with each oxalate. The CL intensity obtained from TCPO-H<sub>2</sub>O<sub>2</sub> was strongest at pH 7.0, whereas the highest intensity with TDPO-H<sub>2</sub>O<sub>2</sub> was at pH 6.5. As the catalytic effect of imidazole in the CL reaction was stronger than that of phosphate buffer which is commonly used as an eluent, the imidazole buffer was selected for the separation of the derivatives. The purity of imidazole has a great influence on both the level of background emission and the variation of baseline noise. Therefore, high-quality imidazole must be used for the preparation of the buffer. The purities of water and the organic modifier such as acetonitrile are also important for high sensitive detection. Thorough mixing of

the effluent from column outlet and CL reagents provides stable baseline and reproducible peaks. Thus, a rotating mixing device is recommended instead of usual T-type mixer. The order of the detection sensitivity was DBD-APy>ABD-APy>NBD-APy. The detection limits of naproxen derivatives of DBD-APy, ABD-APy, and NBD-APy were 0.5, 1.9 and 15 fmol, respectively. The determinations of DL-ibuprofen in rat urine and plasma were carried out as one of the application. The resulting diastereomers derived from DL-ibuprofen were completely separated without interference of endogenous substances.

## REAGENTS FOR CHIRAL AMINES

There are many bioactive chiral amines in biological systems, and thus the determination is one of important theme as same as the determination of chiral carboxylic acids. The derivatization of chiral amines easily performed with the coupling reaction of carboxylic acid. For the resolution of chiral amines, the enantiomers of DBD-Pro and NBD-Pro were synthesized from the reaction of DL-proline with DBD-F or NBD-F (Fig. 2) [12,13]. The diastereomers derived from chiral amines and DBD-Pro or NBD-Pro were separated on reversed-phase chromatography. However, the *R<sub>s</sub>* values were not good enough for real sample analysis. To increase the separation efficiency, DBD- -Pro was developed (Fig. 2) [14]. The resulting peaks were well



**Fig. (2).** Structures of fluorescent chiral derivatization reagents for amines.

separated on reversed-phase ODS column. DBD-OH-Pro derivatives, obtained from the reaction of DBD-F with *cis*-4-hydroxyl-D(or L)-proline, *trans*-4-hydroxy-L-proline, and *trans*-3-hydroxy-L-proline have also been developed for the resolution of chiral amines (Fig. 2) [15]. The resolution efficiency of the derivatives varied in chiral amines tested. DBD-*cis*-4-hydroxyl-D(or L)-proline was recommended to the resolution of *RS*-NEA; while DBD-*trans*-3- hydroxy-L-proline and DBD-*trans*-4-hydroxy-L-proline were suitable for the resolution of DL-PAME. The results demonstrated that the stereostructure, steric hindrance, hydrophobicity and hydrogen bonding of the diastereomer, etc. were affecting the separation. However, the detailed separation mechanism is not obvious because the interaction among the diastereomer, mobile phase and stationary phase in flow system is very complicating.

Isothiocyanate type reagents also react with primary and secondary amines to produce thiocarbamoyl derivatives. PITC is a famous reagent for amino acid sequence analysis in peptide residue. Based upon the structure, DBD-PyNCS and NBD-PyNCS were synthesized from the reaction of  $\text{CSCl}_2$  with DBD-APy or NBD-APy (Fig. 2) [16]. The reaction essentially required heating treatment, and thus, the racemization was possible to occur during the reaction. Judging from the direct resolution using CSP column, the racemization was negligible, and the optical purities were more than 99.5% for all the reagent enantiomers. These reagents exhibited excellent stability, not only as the solid, but also in solution. No significant degradation was observed in acetonitrile solution after storage for 2 weeks at room temperature and 1 month at 5 °C in a refrigerator.

The chiral reagents reacted with primary and secondary amino functional groups in the presence of TEA to produce the corresponding fluorescent thioureas [17]. Instead of TEA, 0.5% quinuclidine, 0.5% DBU, and 0.05 M borate buffer (pH10) are also usable as the activation reagent. The derivatization of this type reagents and chiral amines (e.g. PEA and NEA) effectively proceeded at 55°C for 20 min in the presence of TEA. The resulting diastereomers were well separated on reversed-phase liquid chromatography.

The procedure was applicable to the resolution of a pair of enantiomers of primary and secondary amines, including amino acids [18], peptides [19,20], and some drugs (e.g.,  $\beta$ -blockers) [21].  $\beta$ -Blockers with the *iso*-propylamino moiety (i.e., oxoprenolol, propranolol, alprenolol, atenolol, indenolol, and pindolol) can be detected at lower levels (16-320 fmol) than  $\beta$ -blockers with *tert*-butylamino moiety (i.e., bupranolol, bucamolol, carteolol, timolol) (1.25-8.0 pmol). The lower sensitivity may be due to low derivatization yield because of steric hindrance around the reaction site of the  $\beta$ -blockers. Consequently, care should be taken when tagging to such hindered compounds.

The determination of *RS*-propranolol in rat plasma and saliva after oral administration (10mg/kg) were performed as one of the application [21]. Only *R*-propranolol was detected from the samples obtained from *R*-isomer administration. On the other hand, not only *S*-propranolol but also *R*-propranolol appeared on the chromatograms of the samples of *S*-propranolol administration. Judging from the observation, *S*-propranolol might be converted to *R*-isomer in biological system.

*R*-DBD-PyNCS was used for the separation and detection of DL-amino acids [18,19,22]. The  $R_s$  values of amino acids were in the range 0.55-3.57 for the diastereomers obtained from NBD-PyNCS, and 0.68-2.57 for those from DBD-PyNCS. The  $R_s$  values obtained from neutral and aromatic amino acids were larger than those of basic and acidic amino acids. The diastereomers corresponding to the *R* configuration eluted faster than those of the *S*-configuration with *R*-enantiomer of the reagent. The opposite elution order was obtained from the use of the *S*-enantiomer of the reagents. Since total separation of 18 DL-amino acids in single chromatographic run was fairly difficult, the separation was carried out after divided two, hydrophilic and hydrophobic DL-amino acids. -Ala and 6-amino-*n*-caproic acid were used as IS for hydrophilic (i.e., His, Arg, Ser, Thr, Gly, Glu, Asp, Ala, and Pro) and hydrophobic (Tyr, Val, Met, Ile, Leu, Phe, Trp, and Lys) amino acids, respectively. Best elution profile for the separation of hydrophilic amino acids was by an isocratic elution with water-30 % methanol in acetonitrile (72:28) containing 0.1 % TFA as eluent. That of hydrophobic amino acids was by linear gradient elutions using 25 mM sodium acetate (pH 5.2) and acetonitrile [18].

D-amino acids in human urine and some foodstuffs were determined under the recommended separation and detection conditions [22]. Relative high concentration of D-serine was detected in human urine and the result was similar to a previous report. A couple of D-amino acids, i.e. D-aspartic acid, D-glutamic acid and D-alanine, also appeared on the chromatograms of fermented foodstuffs. These peaks were identified by the use of the opposite enantiomer of DBD-PyNCS (*S*-isomer). The elution order of the peaks was changed by the derivatization with *S*-DBD-PyNCS. Furthermore, the peaks considered to be D- and L-amino acid derivatives were identified by HPLC-ESI-MS.

The maximal excitation and emission wavelengths of the diastereomers obtained from NBD-PyNCS were 490nm and 530 nm, respectively. The diastereomers were also separated by CE and determined with LIF detection [23]. DL-Amino acids derived from NBD-PyNCS were sensitively detected at 50 nM by the CE-LIF. The method was applied to the determination of D-proline and D-aspartic acid in rabbit plasma.

Peptide mixture were also analyzed by the reagents as same as DL-amino acids [19]. DL-Amino acid composition derived from hydrolysate of peptide seemed to be possible by the method using the isothiocyanate reagents. However, the analysis was difficult because the racemization occurred during both vapor-phase hydrolysis using 7M HCl and 10% TFA and liquid-phase hydrolysis using constant-boiling HCl. The racemization ratio of vapor-phase procedure was higher than liquid-phase hydrolysis. Furthermore, the racemization was depending upon the amino acid sequence in peptides. Since the content of D-amino acids is generally very low, the composition analysis of D-amino acids in peptide was difficult by the method.

Edman degradation method utilizing PITC is well known as the sequential analysis of *N*-terminal amino acid in peptide. Since PITC is an achiral compound, the resolution of DL-amino acids in peptide sequence is impossible. In contrast, DBD-PyNCS and NBD-PyNCS are chiral reagents,

and thus amino acid configuration in peptide sequence was resolved by the Edman degradation method [24-27].

The Edman degradation method for sequential analysis of peptide with the isothiocyanate type reagents is divided into following four steps; (I) labeling of *N*-terminal amino group, (II) cleavage of labeled *N*-terminal amino acid, (III) cyclization of liberated amino acid to thiazolinone derivative, and (IV) re-cyclization to thiohydantoin derivative. Since the conversion of unstable thiazolinone to thiohydantoin is very fast, the (II)-(IV) reactions simultaneously proceed in short time. The *N*-terminal amide bond of peptide is cleaved in step (II), and the thiocarbonyl derivative of *N*-terminal amino acid is liberated with the reaction. After these steps, the residual peptide that is a *N*-terminal amino acid shorter than former peptide is produced. The yield in next cycle depends upon the recovery ratio of the peptide. The yields at every step (e.g., yield of labeling, efficiency of the extraction of thiohydantoin, and loss of peptide during the extraction) dictate the detection limit and measurable cycle numbers of analyte peptide. The thiocarbonyl-amino acid is converted to the corresponding thiohydantoin derivative of *N*-terminal amino acid via the thiazolinone. Long heating time is not only decreasing the production, but also increasing the racemization. The degree of racemization is depending on the stereostructure of the peptide analyzed. Thus, it is not easy to predict the degree of racemization ratios of amino acids in every peptide.

The simultaneous separation of all pairs of the thiohydantoin derivatives of DL-amino acids was difficult by single chromatographic run, even though the using of the gradient elutions. Therefore, optimal isocratic elutions toward each pair were used for the resolution of DL-amino acids.

Strong acid (e.g. TFA) treatment is essential for the cleavage of the derivative to thiocarbonyl amino acid, the cyclization from thiocarbonyl to thiazolinone derivatives, and the conversion to thiohydantoin derivative. The racemization during the cyclization and the conversion reactions was inevitable. Therefore, a pair of peaks corresponding to DL-amino acid derivatives appeared on the chromatograms. Since the racemization ratio was usually less than 30%, the resolution of DL-amino acids was easy. When the proposed procedure was applied to some peptide including D-amino acid, the resolution of peptide having 10 residues was successfully performed [26].

The proposed degradation method using DBD-PyNCS was also adopted auto-analysis by a gas-phase sequencer. The resolution of the derivatives was possible without any change of the method using PITC as the tagging reagent [27]. Thus, the sensitivity was not so good owing to the detection at UV 254nm. FL and LIF detection are recommended for the resolution of trace amount of DL-amino acid residues in peptide.

Imai *et al.* [28,29] also determined the DL-amino acids in peptide sequence by Edman degradation method. According to the procedures, DL-amino acids derivatives obtained from Edman reaction using an achiral reagent (NBD-NCS or DBD-NCS) were initially separated by ODS column. In this stage, the species of amino acids were identified. Each amino

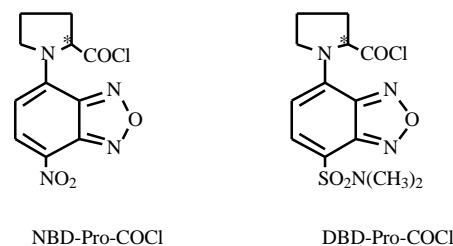
acid was fractionated by column-switching technique and then separated by CSP column.

In spite of development of a lot of fluorescence labeling reagents, the fluorescence reagent for the separation and detection of chiral thiols is very rare. Chiral thiols labeled with OPA and L-valine were separated and detected by reversed-phase chromatography [30].

A pair of thiol enantiomers also reacts with DBD-PyNCS in the presence of pyridine to produce the corresponding dithiocarbamate diastereomers. Several thiols, e.g. DL-cysteine, *RS*-thiopronin and (+)(-)-2-mercaptopropionic acid, were efficiently separated by ODS column with water-acetonitrile containing 0.1 % TFA [31,32]. The *R<sub>s</sub>* values were in the range of 1.05-3.33 for the diastereomers obtained with *R*-DBD-PyNCS. The detection limits were 0.4-2.4 pmol. Since the chiral reagents label not only thiol but also amines under similar reaction conditions, care should be taken in the derivatization of analytes containing both thiol and amino functional groups in the structure because it is possible to transfer the FL moiety from *S* to *N*.

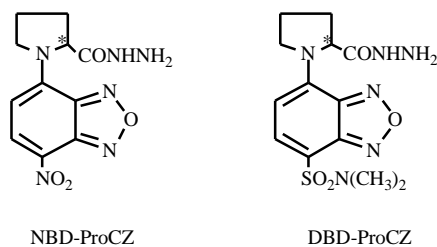
### REAGENTS FOR CHIRAL ALCOHOLS

The labeling of alcoholic OH group is fairly difficult because alcohols are neutral compounds. The derivatization of alcohols is usually carried out by the reaction with carboxylic acid. However, drastic conditions, e.g. long time at reflux, are essentially required for the ester formation. Therefore, the carboxylic acid for the labeling of alcohol is usually converted to its acid chloride (activation form) and then used for the labeling. As benzofurazan derivatives, DBD-Pro-COCl and NBD-Pro-COCl were synthesized from the reaction of DBD-Pro and NBD-Pro with PCl<sub>5</sub> (Fig. 3) [33-35].



**Fig. (3).** Structures of fluorescent chiral derivatization reagents for alcohols.

The chiral reagents were stable as solids. Although the acyl chloride group exhibits excellent reactivity with alcohols, the reagents also react with moisture. Thus, the reagents must be prepared just prior to use. The derivatization in a hydrophobic solvent gave higher yield than in hydrophilic solvent (benzene>THF>acetonitrile). The benzene used in the reaction should be thoroughly dried with suitable reagents such as molecular sieves and sodium wire. The derivatization reaction proceeded even in the absence of the pyridine in the medium. However, the reaction rate was accelerated by the addition of pyridine. Although quinuclidine and TEA also accelerated the derivatization reaction, their efficiencies were approximately one-fourth that of pyridine.



**Fig. (4).** Structures of fluorescent chiral derivatization reagents for ketones.

The derivatives were also stable for at least 240 min at 80 °C. The reactivities of a pair of chiral reagent enantiomers were essentially the same for both enantiomers of heptan-2-ol, representative as chiral alcohol. The FL intensity (detected as a peak area) of the diastereomer obtained from *R*-reagent and *S*-alcohol (or *S*-reagent and *R*-alcohol) was slightly higher than that of *RR* (or *SS*) diastereomer. The resulting ester diastereomers were well separated by normal-phase chromatography, however, the separation was insufficient in reversed-phase chromatography.

DBD-Pro-COCl was more suitable than NBD-Pro-COCl by normal-phase chromatography. However, the derivatives obtained from alcohols and NBD-Pro-COCl can be separated by reversed-phase chromatography. When *S*-enantiomer was used as the chiral derivatization reagent, the corresponding *S*-enantiomers of the alcohols eluted more rapidly than the *R*-enantiomers. The *R<sub>s</sub>* values of the derivatives, obtained from NBD-Pro-COCl and DBD-Pro-COCl, by normal-phase chromatography were 3.0-4.1 and 3.3-4.5, respectively.

These reagents reacted with not only alcohols but also amines to produce corresponding amides [34,35]. The reaction proceeded rather mild conditions than that of alcohols. Good separation of the amide diastereomers was observed in normal-phase chromatography. The separation in reversed-phase chromatography was less than that of ester diastereomers derived from alcohols. Thus, DBD-Pro-COCl and NBD-Pro-COCl could not recommend as the reagents for the resolution of chiral amine. The derivatives were detected with not only conventional fluorescence detector but also laser-induced fluorescence detection. The detection limits of the LIF method reached to 10-50 fmol. The sensitivity with LIF detection was improved by two-orders of magnitude and attomole level detection was possible.

## REAGENTS FOR CHIRAL KETONE

Biologically important carbonyl compounds such as steroids are shown in our body. However, the reagent resolving chiral ketones is very rare. (+)-2,2,2-trifluoro-1-phenylethylhydrazine was only reported for the resolution of chiral ketones, which was the derivatization reagent for GC analysis. According to the reagent structure, DBD-ProCZ and NBD-ProCZ were synthesized from the reaction of hydrazine and DBD-Pro-COCl or NBD-Pro-COCl (Fig. 4) [36]. The reagents reacted with chiral ketones at 65°C for 10 min in the presence of TCA to produce the hydrazone derivatives. The separation of a pair of diastereomers was insufficient for the resolution of chiral ketones in both reversed-phase and normal-phase chromatography. Of

course, the reagents label achiral ketones and aldehydes [37]. As an application of DBD-ProCZ, aldehydes in perfume were determined without any interference.

## CONCLUSION

Although many fluorescent chiral derivatization reagents for the resolution of various chiral molecules are reported, this review focused on benzofurazan bearing chiral reagents. The synthesis, characteristics and application of the fluorescence labeling reagents, which have been developed for the resolution of chiral molecules in HPLC, are outlined. The benzofurazan chiral fluorescent reagents react with target compounds to produce corresponding diastereomers under mild conditions. The resulting derivatives are stable and emit light in long wavelength region. The handling of the reagents is relatively easy, and many of the reagents are now on a market.

Although the FL detection in HPLC provides excellent sensitivity and selectivity, the sensitivity is often insufficient for trace determination in real samples. LIF detection is adopted in such case. Some fluorophores emit light on chemical reaction without the need of optical excitation with lamps such as xenon arc. As the flicker noise based upon the lamp is negligible, extremely high sensitivity is theoretically obtained from CL derived from chemical reaction. Indeed, trace analysis at attomole levels has been achieved with this technique in the reaction of CL reagents (oxalates and H<sub>2</sub>O<sub>2</sub>). The CL method is also possible to apply the sensitive detection of distereomers derived from FL chiral tagging reagents.

As fluorometry is both sensitive and selective, many FL tagging reagents are applied to the analysis of real samples. The FL properties of the resulting derivatives tend to be greatly affected by temperature, viscosity of the solvent, and pH of the medium etc. It should be also noted that undesirable FL materials contaminated in tested samples, especially in biological specimens. Thus, the pre-treatment of real samples is another important topic in trace analysis. In the analysis of real samples such as biological, environmental, and food, most significant and major part of the procedure involves how effectively to obtain trace analytes from complicated matrix. Sample pre-treatment, i.e. clean-up and concentration of analytes, is inevitable for HPLC measurement with derivatization.

## ABBREVIATIONS

|                   |   |   |
|-------------------|---|---|
| ABD-APy           | = | 4-(3-aminopyrrolidin-1-yl)-7-aminosulfonyl-2,1,3-benzoxadiazole |
| ABD-F             | = | 4-aminosulfonyl-7-fluoro-2,1,3-benzoxadiazole                   |
| APy               | = | 3-aminopyrrolidine  |
| Benzo furazan     | = | 2,1,3-benzoxadiazole  |
| CE                | = | electrophoresis   |
| CL                | = | chemiluminescence   |
| CSCl <sub>2</sub> | = | thiophosgene  |
| CSP               | = | chiral stationary phase   |

|                               |   |   |
|-------------------------------|---|---|
| CV                            | = | coefficient of variation  |
| DBD-APy                       | = | 4-(3-aminopyrrolidin-1-yl)-7-( <i>N,N</i> -dimethylaminosulfonyl)-2,1,3-benzoxadiazole          |
| DBD-Pro                       | = | 4-prolyl-7-( <i>N,N</i> -dimethylaminosulfonyl)-2,1,3-benzoxadiazole                            |
| DBD-F                         | = | 4-( <i>N,N</i> -dimethylaminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole                            |
| DBD-NCS                       | = | 4-isothiocyanato-7-( <i>N,N</i> -dimethylaminosulfonyl)-2,1,3-benzoxadiazole                    |
| DBD-OH-Pro                    | = | 4-hydroxylpropyl-7-( <i>N,N</i> -dimethylaminosulfonyl)-2,1,3-benzoxadiazole                    |
| DBD-Pro-COCl                  | = | 4-(2-chloroformylpyrrolidin-1-yl)-7-( <i>N,N</i> -dimethylaminosulfonyl)-2,1,3-benzoxadiazole   |
| DBD-Pro                       | = | 4-prolyl-7-( <i>N,N</i> -dimethylaminosulfonyl)-2,1,3-benzoxadiazole                            |
| DBD-ProCZ                     | = | 4-(2-carbazolylpyrrolidin-1-yl)-7-( <i>N,N</i> -dimethylaminosulfonyl)-2,1,3-benzoxadiazole     |
| DBD-PyNCS                     | = | 4-(3-isothiocyanatopyrrolidin-1-yl)-7-( <i>N,N</i> -dimethylaminosulfonyl)-2,1,3-benzoxadiazole |
| DBU                           | = | 1,8-diazabicyclo[5.4.0]undecene   |
| DPDS                          | = | 2,2'-dipyridyl disulfide  |
| EDC                           | = | 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide   |
| ESI                           | = | electrospray ionization   |
|                               | = | fluorescence quantum yield  |
| FL                            | = | fluorescence  |
| GC                            | = | gas chromatography  |
| H <sub>2</sub> O <sub>2</sub> | = | hydrogen peroxide   |
| HPLC                          | = | high-performance liquid chromatography  |
| LIF                           | = | laser-induced fluorescence  |
| MS                            | = | mass spectrometry   |
| NBD-APy                       | = | 4-(3-aminopyrrolidin-1-yl)-7-nitro-2,1,3-benzoxadiazole   |
| NBD-F                         | = | 4-nitro-7-fluoro-2,1,3-benzoxadiazole   |
| NBD-NCS                       | = | 4-isothiocyanato-7-nitro-2,1,3-benzoxadiazole   |
| NBD-Pro-COCl                  | = | 4-(2-chloroformylpyrrolidin-1-yl)-7-nitro-2,1,3-benzoxadiazole                                  |
| NBD-ProCZ                     | = | 4-(2-carbazolylpyrrolidin-1-yl)-7-nitro-2,1,3-benzoxadiazole                                    |

|                  |   |  |
|------------------|---|--|
| NBD-PyNCS        | = | 4-(3-isothiocyanatopyrrolidin-1-yl)-7-nitro-2,1,3-benzoxadiazole |
| NEA              | = | 1-(1-naphthyl)ethylamine   |
| ODS              | = | octadecyl silica gel   |
| OPA              | = | orthophthalaldehyde  |
| PAME             | = | phenylalanine methyl ester                                       |
| PCl <sub>5</sub> | = | phosphorus pentachloride   |
| PEA              | = | 1-phenylethylamine   |
| PITC             | = | phenylisothiocyanate   |
| Rs               | = | resolution   |
| TCA              | = | trichloroacetic acid   |
| TCPO             | = | bis(2,4,6-trichlorophenyl)oxalate                                |
| TDPO             | = | bis[4-nitro-2-(3,6,9-trioxadecyloxy)phenyl]oxalate               |
| TEA              | = | triethylamine  |
| TFA              | = | trifluoroacetic acid   |
| THF              | = | tetrahydrofuran  |
| TPP              | = | triphenylphosphine   |
| UV               | = | ultraviolet  |
| VIS              | = | visible  |

## REFERENCES

- Gübitz, G.; Schmid, M.G. *Methods in Molecular Biology*, Vol. 243. *Chiral Separations Methods and Protocols*, Humana Press: Totowa, New Jersey, **2004**.
- Toyo'oka, T. In *Modern Derivatization Methods for Separation Sciences*, Toyo'oka, T., Ed.; Derivatization for resolution of chiral compounds, Wiley, Chichester, UK, **1999**; pp 217-289.
- Toyo'oka, T. *Biomed. Chromatogr.*, **1996**, *10*, 265.
- Sun, X.X.; Sun, L.Z.; Aboul-Enein, H.Y. *Biomed. Chromatogr.*, **2001**, *15*, 116.
- Srinivas, N.R. Igwemezie, L.N. *Biomed. Chromatogr.*, **1992**, *6*, 163.
- Srinivas, N.R. *Biomed. Chromatogr.*, **2004**, *18*, 207.
- Chou, T.Y.; Gao, C.X.; Grinberg, N.; Krull, I.S. *Anal. Chem.*, **1989**, *61*, 1548.
- Toyo'oka, T.; Ishibashi, M.; Terao, T. *Analyst*, **1992**, *117*, 727.
- Toyo'oka, T.; Ishibashi, M.; Terao, T. *Anal. Chim. Acta*, **1993**, *278*, 71.
- Toyo'oka, T.; Ishibashi, M.; Terao, T. *J. Chromatogr.*, **1992**, *625*, 357.
- Toyo'oka, T.; Ishibashi, M.; Terao, T. *J. Chromatogr.*, **1992**, *627*, 75.
- Toyo'oka, T.; Suzuki, T.; Saito, Y.; Uzu, S.; Imai, K. *Analyst*, **1989**, *114*, 1233.
- Al-Kindy, S.; Santa, T.; Fukushima, T.; Homma, H.; Imai, K. *Biomed. Chromatogr.*, **1998**, *12*, 276.
- Min, J.Z.; Toyo'oka, T.; Kato, M.; Fukushima, T. *Biomed. Chromatogr.*, **2004**, in press.
- Min, J.Z.; Toyo'oka, T.; Fukushima, T.; Kato, M. *Anal. Chim. Acta*, **2004**, *515*, 243.
- Toyo'oka, T.; Liu, Y.-M. *Analyst*, **1995**, *120*, 385.
- Toyo'oka, T.; Liu, Y.-M. *J. Chromatogr. A*, **1995**, *689*, 23.
- Jin, D.; Nagakura, K.; Murofushi, S.; Miyahara, Y.; Toyo'oka, T. *J. Chromatogr. A*, **1998**, *822*, 215.
- Liu, Y.-M.; Toyo'oka, T. *Chromatographia*, **1995**, *40*, 645.
- Liu, Y.-M.; Miao, J.-R.; Toyo'oka, T. *Anal. Chim. Acta*, **1995**, *314*, 169.
- Toyo'oka, T.; Toriumi, M.; Ishii, Y. *J. Pharm. Biomed. Anal.*, **1997**, *15*, 1467.

- [22] Jin, D.; Miyahara, Y.; Oe, T.; Toyo'oka, T. *Anal. Biochem.*, **1999**, 269, 124.
- [23] Liu, Y.-M.; Schneider, M.; Sticha, C.M.; Toyo'oka, T.; Sweedler, J.V. *J. Chromatogr. A*, **1998**, 800, 345.
- [24] Toyo'oka, T.; Suzuki, T.; Watanabe, T.; Liu, Y.-M. *Anal. Sci.*, **1996**, 12, 779.
- [25] Suzuki, T.; Watanabe, T.; Toyo'oka, T. *Anal. Chim. Acta*, **1997**, 352, 357.
- [26] Toyo'oka, T.; Tomoi, N.; Oe, T.; Miyahara, T. *Anal. Biochem.*, **1999**, 276, 48.
- [27] Toyo'oka, T.; Jin, D.; Tomoi, N.; Oe, T.; Hiranuma, H. *Biomed. Chromatogr.*, **2001**, 15, 56.
- [28] Matsunaga, H.; Santa, T.; Hagiwara, K.; Homma, H.; Imai, K.; Uzu, S.; Nakashima, K.; Akiyama, S. *Anal. Chem.*, **1995**, 67, 4276.
- [29] Toriba, A.; Santa, T.; Iida, T.; Imai, K. *Analyst*, **1999**, 124, 43.
- [30] Sano, A.; Takitani, S.; Nakamura, N. *Anal. Sci.*, **1995**, 11, 299.
- [31] Jin, D.; Takehana, K.; Toyo'oka, T. *Anal. Sci.*, **1997**, 13, 113.
- [32] Jin, D.; Toyo'oka, T. *Analyst*, **1998**, 123, 1271.
- [33] Toyo'oka, T.; Ishibashi, M.; Terao, T.; Imai, K. *Analyst*, **1993**, 118, 759.
- [34] Toyo'oka, T.; Liu, Y.-M.; Hanioka, N.; Jinno, H.; Ando, M. *Anal. Chim. Acta*, **1994**, 285, 343.
- [35] Toyo'oka, T.; Liu, Y.-M.; Hanioka, N.; Jinno, H.; Ando, M.; Imai, K. *J. Chromatogr. A*, **1994**, 675, 79.
- [36] Toyo'oka, T.; Liu, Y.-M. *Anal. Proc.*, **1994**, 31, 265.
- [37] Toyo'oka, T.; Liu, Y.-M. *J. Chromatogr. A*, **1995**, 695, 11.