*Journal of Pharmaceutical & Biomedical Analysis*
Vol. 7, No. 12, pp. 1395–1403, 1989
Printed in Great Britain

0731–7085/89 $3.00 + 0.00


# Fluorogenic reagents, having benzofurazan structure, in liquid chromatography*

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**Abstract**: Newly synthesized fluorescent reagents, NBD-F and PBD-$SO_2$F for amines, DBD-F for thiols and amines, and ABD-F and SBD-F for thiols are reviewed in terms of their reactivity, fluorescence characteristics, stability, selectivity, and their applicability to analysis.

**Keywords**: *Benzofurazan; fluorogenic reagents; HPLC; fluorescent derivatization; amine; thiol.*

## Introduction

Liquid chromatography (LC) combined with fluorescence detection is one of the most suitable tools for the determination of trace quantities of hormones, amines, amino acids, peptides, proteins, carbohydrates, lipids, nucleic acids or drugs. However, many of these substances do not fluoresce and for sensitive (sub-pmol) and specific detection of an amino, hydroxy, phenol, carbonyl, carboxy or mercapto group, derivatization may be necessary. Many fluorogenic reagents have been developed and are widely used in pre- and post-column procedures.

Recently, several fluorogenic reagents having halogenobenzofurazan structure have been synthesized. These reagents include 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) [1, 2], ammonium-7-fluoro-2,1,3-benzoxadiazole-4-sulphonate (SBD-F) [3], 4-(aminosulphonyl)-7-fluoro-2,1,3-benzoxadiazole (ABD-F) [4], 4-(*N*,*N*-dimethyl-aminosulphonyl)-7-fluoro-2,1,3,-benzoxadiazole (DBD-F) [5] and 4-(chlorosulphonyl)-7-fluoro-2,1,3-benzoxadiazole (CBD-F) [4]. Two other reagents having a similar structure were 4-(fluorosulphonyl)-7-phenoxy-2,1,3-benzoxadiazole (PBD-$SO_2$F) and 4-(fluorosulphonyl)-7-benzyloxy-2,1,3-benzoxadiazole (BBD-$SO_2$F) [6] (Fig. 1).

In this paper the feature of these reagents, except CBD-F, are discussed in terms of their reactivity, fluorescence characteristics, stability, selectivity, and their applicability to analysis.

For more details, the readers are referred to refs 7–10.

*Presented at the "Third International Symposium on Drug Analysis", May 1989, Antwerp, Belgium.
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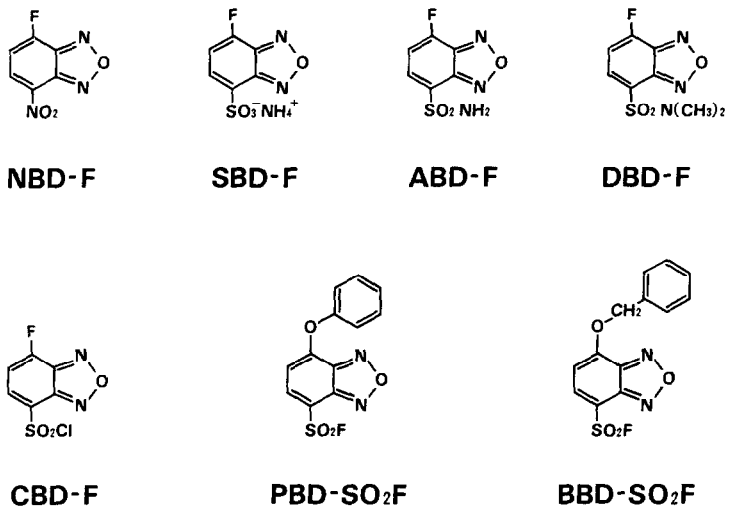


**Figure 1**
Fluorogenic reagents having benzofurazan structure.

## History

The usefulness of the benzofurazan structure for the fluorometric analysis of substances containing the amino group was first demonstrated by 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) [11] which was used for the determination of hydroxyproline and proline in blood plasma [12]. NBD-Cl reacts especially well with secondary amines such as proline under a relatively drastic conditions (pH 9.5 and 60°C for 3 min) to give high fluorescence at 540 nm with excitation at 470 nm. These excitation and emission wavelengths are preferable for the determination of amines in the biological samples, which contain many interfering substances having fluorescence of short wavelengths around 300 nm. To improve the reactivity, a new reagent, NBD-F, in which the chloro moiety of NBD-Cl was replaced with an atom of smaller diameter, fluorine, was proposed [1]. As expected, NBD-F was 50–100 times more reactive for amines than NBD-Cl. Subsequently, the other reagents having 4-fluorobenzofurazan structure, DBD-F, ABD-F and SBD-F have been introduced [3–5]. In the course of their development, PBD-$SO_2F$ and BBD-$SO_2F$ have been also synthesized [6].

## Fluorobenzofurazan

NBD-F, DBD-F, ABD-F and SBD-F react with various nucleophiles (e.g. thiols, amines, phenols and alcohols) and their order of reactivities (NBD-F>DBD-F>ABD-F>SBD-F) reflect the intensities of electron withdrawal at the 7-position of the 4-fluorobenzofuran. According to the literature [13, 14], the Hammett constants for nitro, sulphonamide and sulphonic acid substitutes are 0.78, 0.62 and 0.33, respectively, but that for dimethylsulphonamide ($SO_2NMe_2$) has not been established. Considering the recent observation that DBD-F is more reactive than ABD-F for thiol and amine, the electron withdrawing activity of dimethylsulphonamide may be larger than that of sulphonamide group [5].

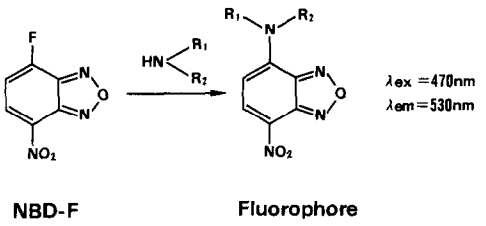


**Figure 2**
Reaction of NBD-F with amines.

*NBD-F*

*Reactivity of amines.* NBD-F reacts with primary and secondary amines to give fluorescent adducts that fluoresce at 530 nm with excitation at 470 nm (Fig. 2). The reaction was thought to proceed via a Meisenheimer complex [15] and was affected by various factors such as pH, temperature, organic solvent and buffer [16]. Increases in the pH, temperature and proportion of organic solvent in the reaction medium increase the reaction rate. Ethanol or acetonitrile and borate are the preferred co-solvent and buffer species for this reaction.

One minute is sufficient for the complete reaction of amines, amino and imino acids with a few mM NBD-F in 50% acetonitrile or ethanol–0.1 M borate buffer (pH 8.0) at 60°C in the dark. These conditions are sufficient for the complete reaction for the least reactive amino acid, aspartic acid. Usually, hydrochloric acid is added to terminate the reaction and suppress the blank.

The pseudo-first-order reaction rate constant of glycine with excess NBD-F is $1.7 \times 10^{-1}$ $min^{-1}$ under the condition at pH 8.0 and 40°C.

*Fluorescence characteristics of NBD-amines.* NBD-amines fluoresce at 520–540 nm with excitation at 450–470 nm [17, 18]. The quantum efficiencies of NBD-hydroxyproline range from 0.01 in water (pH 9) to 0.80 in isobutyl methyl ketone [19], suggesting that the addition of organic solvents is desirable for the sensitive detection of NBD-amines. The hydrolysis product of NBD-F, namely 4-hydroxy-7-nitrobenzo-2-oxa-1,3-diazole (NBD-OH), fluoresces at 550 nm (excitation at 470 nm) with an intensity that is one-tenth weaker than those of NBD-adducts of amines.

*Stability.* NBD-F is stable in acetonitrile under refrigeration (4°C) for 1 week, but only for 1 day in ethanol.

The resultant NBD-amines are stable in the reaction medium for about 1 week under refrigeration (4°C) in the dark.

*Selectivity.* NBD-F is primarily a reagent for amines, but it reacts with other nucleophiles as described above. The reaction with thiols is performed at acidic pH (e.g. 4.5) to give fluorescence, but their fluorescence intensities (maximum at 540 nm with excitation at 420 nm) are only about 5% of those of NBD-amines [11]. For cysteine, the NBD moiety of S-NBD transfers to an amino group, and N-NBD-cysteine or N-NBD-cystine is produced [20]. The reaction products with phenolic hydroxy groups absorb at 380 nm, but do not fluoresce [11, 18].

*Application to amine determination.* With the notable exception of tryptophan, all the essential amino acids can be analysed after derivatization with NBD-F. The NBD-adduct of tryptophan does not fluoresce unless it is subjected to photochemical or electro-

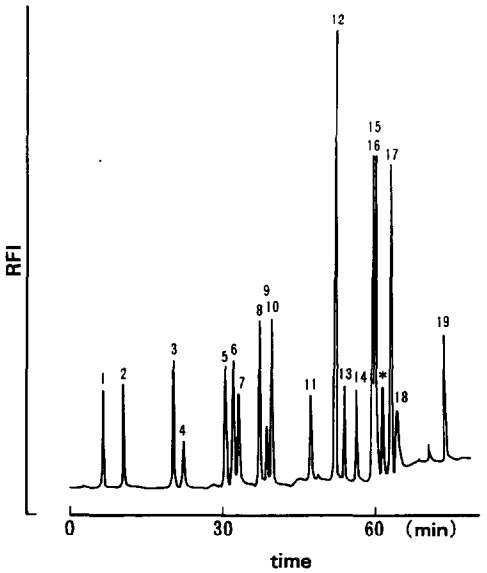


**Figure 3**
Chromatograms of a mixture of 18 NBD amino acids monitored by fluorescence detection, and with an applied potential of +0.48 V [22]. 1, NBD-aspartic acid; 2, NBD-glutamic acid; 3, NBD-serine; 4, NBD-histidine; 5, NBD-glycine; 6, NBD-arginine; 7, NBD-OH; 8, NBD-threonine; 9, NBD-proline; 10, NBD-alanine; 11, NBD-$NH_2$; 12, NBD-valine; 13, NBD-methionine; 14, NBD-cystine; 15, NBD-isoleucine; 16, NBD-leucine; 17, NBD-phenylalanine; 18, NBD-lysine; 19, NBD-tyrosine; *NBD-tryptophan; RFI, relative fluorescence intensity. HPLC conditions: column, TSK gel ODS-80Tm (250 × 4.6 mm, i.d., 5 μm); gradient elution, see ref. 22; fluorescence detection, excitation at 470 nm and emission at 530 nm.

chemical oxidation [21, 22]. Thus, all the essential amino acids in biological fluids can be determined as their NBD-adducts by LC separation with in-line photochemical or electrochemical oxidation and fluorometric detection (Fig. 3). This system has been used in conjunction with automated derivatization for amino acid determination in serum [23]. Care should be taken for the determination of minute amounts of amino acids in biological samples because metal ions such as cuprous and nickel ions interfere with the derivatization reaction. The addition of EDTA·2Na (1 mM) eliminates this source of interference [24].

Reaction mixtures of amino acids, including proline [2, 25, 26], of protein hydrolysates [27, 28], proteins [16], sulphoglutathione in blood [29], biogenic amines [17], hexosamines [30], neutral sugars such as glycamines [31], phenylpropanolamine [32] and selenium as penicillamine selenotrisulphide [33] with NBD-F have been analysed by reversed-phase LC with detection limits <1 pmol. NBD-F has also been used for amino and imino acid determinations with post-column reaction and similar detection limits [34].

*Related reagents.* Alternatives to NBD-F for amine determination are NBD-Cl and 4-alkoxy-7-nitrobenzofurazan (benzo-2-oxa-1,3-diazole) derivatives [19, 35]. However, these reagents are not as reactive as NBD-F [16]. Thus, the derivatization reaction performed with these reagents for the determination of amines such as hydroxyproline in collagen hydrolysate [19], amines derived from nitrosamines [36, 37], or drugs (tripeptide and glybenclamide) [38, 39], in biological fluids [38–40], should be modified to include the more reactive NBD-F.

*DBD-F, ABD-F and SBD-F*

*Reactivity to thiols.* DBD-F, ABD-F and SBD-F react with thiols to give fluorescence at 510 nm with excitation at 380 nm (Fig. 4). DBD-F and ABD-F react with thiols quantitatively at 50°C and pH 8.0 for 10 min. SBD-F required rather drastic conditions (at pH 9.5 and 60°C for 1 h) for the quantitative derivatization.

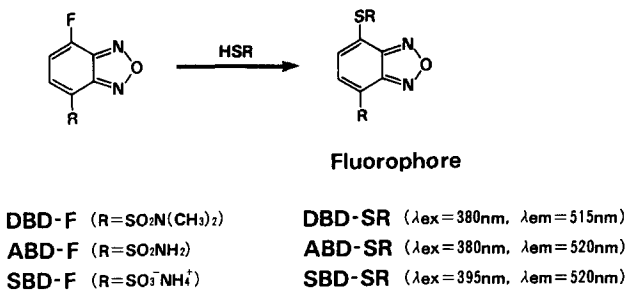


**Figure 4**
Reaction of DBD-F, ABD-F and SBD-F with thiols.

The pseudo-first-order reaction rate constants of homocysteine with excess DBD-F, ABD-F and SBD-F (at pH 7.0 and 60°C) were $3.07 \times 10^{-1}$, $1.16 \times 10^{-1}$ and $3.56 \times 10^{-3}$ $min^{-1}$, respectively.

*Fluorescence characteristics of DBD-, ABD- and SBD-thiols.* DBD-, ABD- and SBD-thiols fluoresce intensely at 510–520 nm (excitation at 380–395 nm). The solvent composition and pH affected the fluorescence intensity and the wavelength of their thiol adducts. They fluoresce highly at pH 2–7 and in organic solvents, especially in ethanol, rather than in water [41]. The hydrolysis products of these reagents have negligible fluorescence.

*Stability.* DBD-F in acetonitrile and ABD-F and SBD-F in acetonitrile or borate buffer (pH 9.5) are stable at room temperature for about 1 week.

*Selectivity.* Generally speaking, DBD-F, ABD-F and SBD-F should be used for the determination of the thiol. Whereas a few percent of proline was labelled with ABD-F and DBD-F under the conditions for the quantitative derivatization of thiols, the sensitivities of the ABD- and DBD-adducts of proline were much lower than that of the thiol adducts when the fluorescence was detected at 510 nm (excitation at 480 nm) for ABD-adducts, 520 nm (excitation at 495 nm) for DBD-adducts.

For the determinations of amine, complete reaction was achieved after 30 min at pH 9.3 and 50°C. However, the reaction of amines with DBD-F was only 78% complete after 2 h, under the same conditions. The pseudo-first-order reaction rate constants for the reaction of excess DBD-F and ABD-F with proline were $6.60 \times 10^{-2}$ (at pH 8.0 and 50°C) and $6.19 \times 10^{-1}$ $min^{-1}$ (at pH 9.3 and 50°C) for DBD-F, and $2.44 \times 10^{-2}$ (at pH 8.0 and 50°C) and $5.80 \times 10^{-1}$ $min^{-1}$ (at pH 9.3 and 50°C) for ABD-F [5].

The fluorescence intensity of DBD-amine adducts was highest at pH 2–3, and was 2.5 times higher than that of the corresponding ABD-amine adducts, but only 25% of that of NBD-amine adducts. The optimum emission wavelengths of DBD-amine and ABD-amine were 594–600 nm (excitation at 466–469 nm) and 600–613 nm (excitation at 466–469 nm), respectively.

The reactivities of SBD-F, ABD-F and DBD-F with methanol were very low, and because these reagents were unstable in the reaction medium, the reaction yields with this analyte were also low. The pseudo-first-order reaction rate constants for ABD-F and SBD-F with methanol were $5.17 \times 10^{-2}$ and $2.16 \times 10^{-2}$ $min^{-1}$, respectively, in 0.15 M

KOH solution at 45°C (unpublished data). The fluorescence intensity of ABD-OMe was 2 times that of SBD-OMe and 200 times that of DBD-OMe.

*Application to the determination of thiols.* DBD-F, ABD-F and SBD-F were suitable for the selective and sensitive detection of low molecular weight thiols such as cysteine and glutathione. ABD-labelled biological thiols such as cysteine and cysteinylglycine in human plasma [42, 43], glutathione in human blood cells [42] and homocysteine in human plasma [43] were separated by reversed-phase LC and detected fluorometrically at levels of 50 fmol to 1 pmol. Cysteine containing peptides were detected by SBD-F in a manual method [44] and the antihypertensive drug, captopril, has also been derivatized with SBD-F and determined by LC [45] (Fig. 5).

The combined use of ABD-F and SBD-F has permitted the simultaneous determination of thiols and disulphides [46], and ABD-F has been used as a differential analytical reagent for proteins.

*Simultaneous determination of thiols and disulphides.* Biological thiols and disulphides in rat and hamster tissues were determined simultaneously by LC with fluorescence detection using ABD-F and SBD-F [46]. The strategy of the method was as follows (Fig. 6). Initially, the thiols in the sample were labelled with ABD-F in an alkaline medium (pH 9.3) containing 5 mM $Na_2EDTA$. After the extraction of both unreacted and hydrolysed ABD-F with ethyl acetate (AcOEt), the remaining disulphides were derivatized with SBD-F in the presence of a reducing agent, *n*-tributylphosphine (TBP). The ABD-thiols derived from the thiols and SBD-thiols derived from the disulphides are separated on a reversed-phase column and detected fluorometrically (excitation at 380 nm, emission at 510 nm). The chromatogram thus obtained from rat liver tissue demonstrates the capability of the simultaneous determination of the reduced and oxidized form of glutathione by this method (Fig. 7).

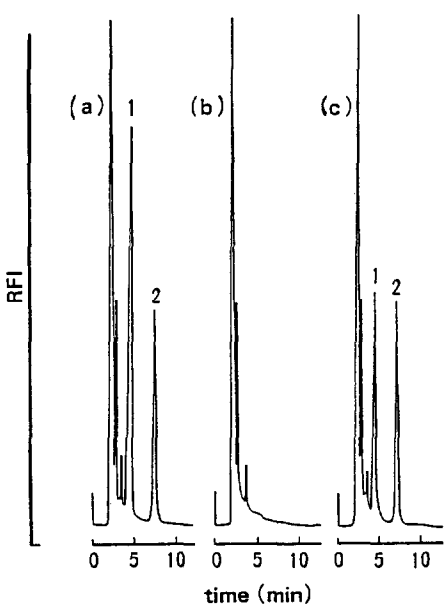


**Figure 5**
Chromatograms of SBD-captopril [45]. (a) standard SBD-adducts (15 ng captopril and 37.5 ng SQ25,233, an internal standard, (b) blank plasma reacted with SBD-F, (c) dog-plasma sample (30 min after oral administration of 50 mg captopril). 1, SBD-captopril; 2, SBD-SQ25,233; RFI, relative fluorescence intensity. LC conditions: column μBondapak $C_{18}$ (300 × 3.9 mm, i.d., 8–10 μm); eluent, methanol–1% phosphoric acid (35:65, v/v); fluorescence detection, excitation at 385 nm and emission at 515 nm.

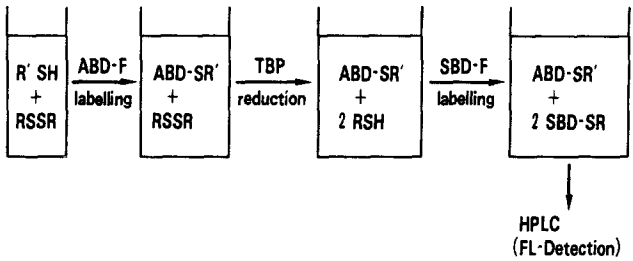


**Figure 6**
Strategy for simultaneous determination of thiols and disulphides. R'SH, thiol; RSSR, disulphide; ABD-SR, ABD-adduct of thiol; TBP, tri-*n*-butylphosphine; RSH, thiol derived from RSSR by reduction; SBD-SR, SBD-adduct of thiol derived from disulphide; FL-Detection, fluorescence detection.

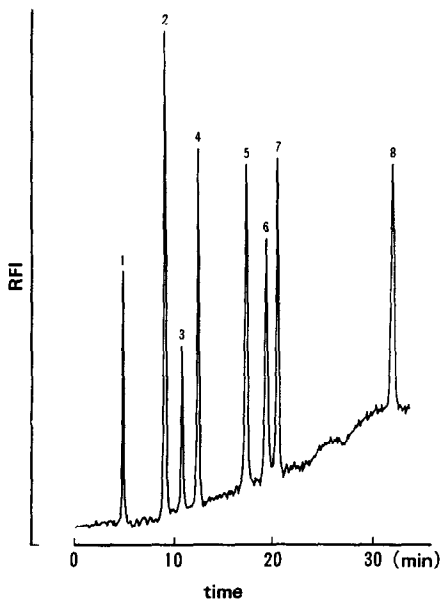


**Figure 7**
LC separation of ABD-thiols and SBD-thiols. 1, SBD-cysteine; 2, SBD-homocysteine; 3, ABD-cysteine; 4, SBD-glutathione; 5, ABD-homocysteine; 6, SBD-*N*-acetylcysteine; 7, ABD-glutathione; 8, ABD-*N*-acetylcysteine; RFI, relative fluorescence intensity. LC conditions: Intersil ODS (150 × 4.6 mm, i.d., 5 μm); column temperature, 40°C; a linear gradient elution from A (0.15 M $H_3PO_4$)–B ($CH_3CN$) (96:4, v/v) to A–B (85:15, v/v) over 30 min and an isocratic elution of A–B (85:15, v/v) for 10 min; fluorescence detection, excitation at 380 nm and emission at 510 nm; flow rate, 1.0 ml $min^{-1}$.

*Differentiation reaction with ABD-F.* In a macromolecule such as protein and nucleic acid there may be several potentially reactive functional groups. For example, egg-white albumin has four cysteine residues. However, the micro-environment of each cysteine residue is not well characterized. Accordingly, egg-white albumin was reacted with ABD-F to determine how many cysteine residues are located at the surface of the molecule [47]. The protein was derivatized [47] with ABD-F under mild conditions (pH 8.0 and 40°C for 1 h) and then subjected to enzymatic hydrolysis. LC separation of the SBD-labelled fragments and amino acid sequence analysis of the fragments revealed that one cysteine residue (367 residue from the N-terminus) was located at the outer surface of the molecule and that the other three residues were located inside the molecule.

*Related reagent.* Ammonium 7-chloro-2,1,3-benzoxadiazole-4-sulphonate (SBD-Cl) is a potential alternative to SBD-F, but it is 30–100 times less reactive.

**Benzofurazansulphonyl Fluorides**

*PBD-$SO_2F$ and BBD-$SO_2F$*

Because of the difficulty of synthesis, there are only limited data on the reactivity of PBD-$SD_2F$ [6], and BBD-$SO_2F$ has yet to be characterized.

The structural resemblance of PBD-$SO_2F$ to *N*,*N*-dimethylaminonaphthalene sulphonyl chloride (DNS-Cl) prompted a comparison of their reactivities. PBD-$SO_2F$ and DNS-Cl both react with thiols and amines producing fluorescent products. However, PBD-$SO_2F$ was hydrolysed faster than DNS-Cl.

PBD-$SO_2F$ reacts with thiols at pH 9.5 and at 60°C to give fluorescence at 500–531 nm with excitation at 372–405 nm, but the adducts are not stable. PBD-$SO_2F$ also reacts with amines to give fluorescence at 565–569 nm (excitation at 452–455 nm; Fig. 8), but hydrolysis of the reagent proceeds faster than the derivatization reaction. The relative fluorescence intensities of the PBD-amine adducts are about twice that of the corresponding DBD-amines.

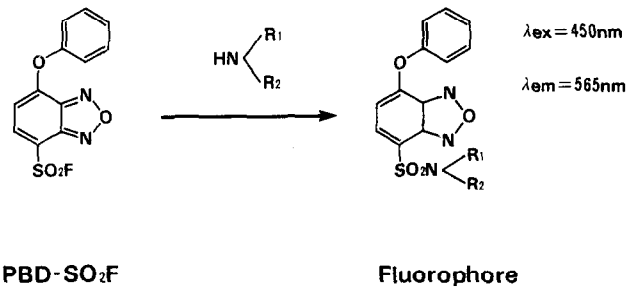


**Figure 8**
Reaction of PBD-$SO_2F$ with amines.

## Conclusions

In this paper, it has been shown that NBD-F is a useful fluorogenic reagent for amines, and DBD-F, ABD-F and SBD-F are useful for the analysis of thiols. Also shown was some utility of DBD-amines, ABD-amines and PBD-amines as fluorescent probes.

Except for the post-column reaction of amino acids with NBD-F [34], the application of these fluorogenic reagents has generally involved pre-column derivatization reactions. However, post-column derivatization is sometimes required for the *in situ* investigation of unstable substances such as conjugates or mixed disulphides. Among the reagents described so far, SBD-F and PBD-$SO_2F$ might be the most appropriate for the post-column derivatization of amines and thiols, because their hydrolysates are only weakly fluorescent and the fluorescence wavelengths of the parent compounds are different from those of the derivatized products.

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[*Received 21 May 1989*]