

# Fluorescence Labeling Reagents

## NBD- and DBD- Series

Estimating trace substances is an important and sometimes difficult task. Fluorescence detection-HPLC is an important method for trace analysis. Many fluorescence labeling reagents have been developed, achieving higher sensitivity and selectivity. NBD- and DBD- derivatives have become popular because these reagents emit strong fluorescence at long wavelengths.

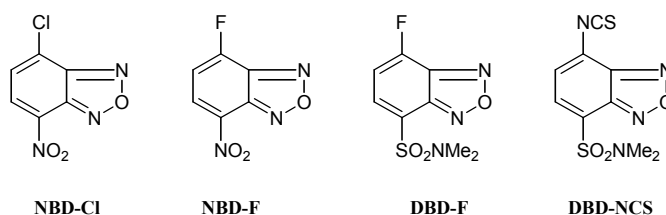
Recently Imai, Toyo'oka and co-workers developed several NBD- and DBD- derivatives. They reported good results for application to amino, mercapto, hydroxy, carbonyl, and carboxyl groups. Usage of the NBD- and DBD- derivatives is described for each functional group.

### Amino Group:

NBD-Cl was the earliest fluorescence reagent which was applied to HPLC and its effectiveness for the secondary amines, such as proline, was reported. Imai and co-workers developed NBD-F<sup>1-3)</sup>, in which the chlorine at the 4-(7-) position was replaced with fluorine for HPLC analysis. They obtained good results. NBD-F has higher reactivity than NBD-Cl. It has a good detection limit for the secondary amines and is satisfactory for primary amines. Also, they developed a DBD-F where a dimethylsulfamoyl group was introduced into the benzoxadiazole structure.

They used it for the analysis of amino acids by reverse phase HPLC. DBD-F<sup>4-6)</sup> is itself non-fluorescent. Amino acids can be detected and analyzed with high sensitivity in sub-pico mole quantities.

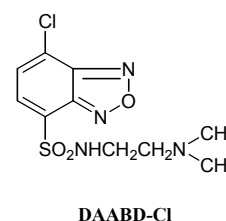
DBD-NCS<sup>7-9)</sup> has an isothiocyanato group at the 4-(7-) position of the DBD structure, is able to be utilized for Edman degradation.



### Mercapto Group:

DBD-F reacts quantitatively with thiols and forms a 4-substituted DBD which emits a strong fluorescence. The detection limits of cystine and glutathione are reported as 0.92 and 0.16 pico mole. Recently, Imai and co-workers have developed a new method for protein analysis using DAABD-Cl. According to the report, the S-S bonds on proteins were first, reductively cleaved to yield the primary proteins. The newly generated SH functional groups of resulting proteins were derivatized by reaction with DAABD-Cl to yield fluorescent labeled protein mixtures.

The fluorescent labeled protein mixtures were separated and isolated by fluorescence HPLC. Next, the isolated protein was digested by protease to obtain the peptide mixtures. The peptide mixtures were analyzed by LC-MS/MS and the resulting mass spectral data were analyzed to identify the original protein by the MASCOT database system. This method is expected to become the new proteome analysis method.<sup>10)</sup>



The fluorescent labeled protein mixtures were separated and isolated by fluorescence HPLC. Next, the isolated protein was digested by protease to obtain the peptide mixtures. The peptide mixtures were analyzed by LC-MS/MS and the resulting mass spectral data were analyzed to identify the original protein by the MASCOT database system. This method is expected to become the new proteome analysis method.<sup>10)</sup>

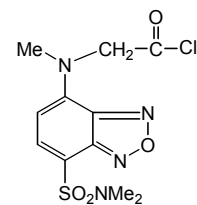
**Keywords** : fluorescence labeling reagents, NBD derivatives, DBD derivatives

2007. May, A-1079E

### Hydroxy Group:

DBD-F does not work well for alcohols and phenols. Imai developed DBD-COCl<sup>11,12</sup> which reacts with a hydroxy group and forms a stable fluorescence adduct. DBD-COCl has a *N*-chloroformylmethyl-*N*-methylamino group at the 4-(7-) position of the DBD structure. It forms a stable adduct with hydroxy, amino and mercapto groups. The adducts can be separated and detected using reverse phase HPLC. The detection limits of androsterone, estrone and mandelic acid are reported as 38, 40 and 125 femto moles.

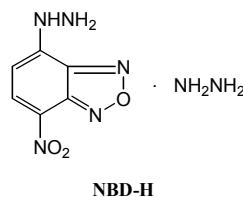
DBD-COCl is a better labeling reagent than the known electrophilic fluorescence reagents for nucleophilic functional groups.



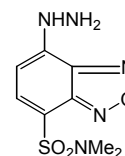
DBD-COCl

### Carbonyl Group:

NBD-H and DBD-H<sup>13</sup>, in which a hydrazino group is introduced at the 4-(7-) position of NBD- and DBD-, were developed for aldehydes and ketones. NBD-H and DBD-H are non fluorescent. However their adducts with aldehydes and ketones emit very strongly. The detection limit of propionaldehydes with DBD-H is reported as 120 femto mole. Generally NBD-H is used for analysis of aldehydes. DBD-H, which more reactive than NBD-H, is used for keto steroids and sugars.



NBD-H

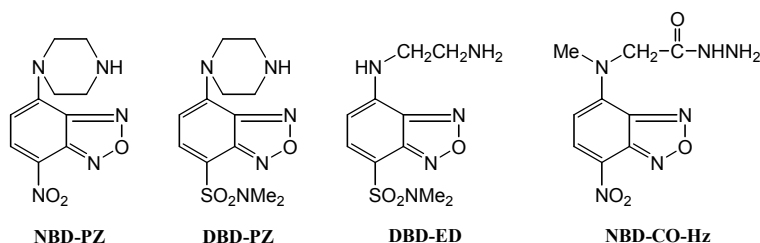


DBD-H

### Carboxyl Groups:

NBD-, DBD-PZ<sup>14</sup> with a piperazine group and DBD-ED<sup>15,16</sup> with 2-aminoethylamino group introduced at the 4-(7-) position of the NBD-, DBD- structure, respectively, are utilized for the fluorescence detection of carboxylic acids. NBD-, DBD-PZ and DBD-ED with carboxylic acids at room temperature in the presence of a condensing agent to yield stable amides. These amides can be separated by reverse phase HPLC for the detection of its emission of fluorescence. The detection limits for saturated carboxylic acids are reported to be 3.2-4.7 femtomols. Applications are expected to include for the detection and quantitative determination of prostaglandins, cholic acid.

NBD-CO-Hz<sup>17</sup> with *N*-hydrazinocarbonylmethyl-*N*-methylamino group introduced at the 4-(7-) position of the NBD- structure is also used to fluorescence detection of carboxylic acids.



NBD-PZ

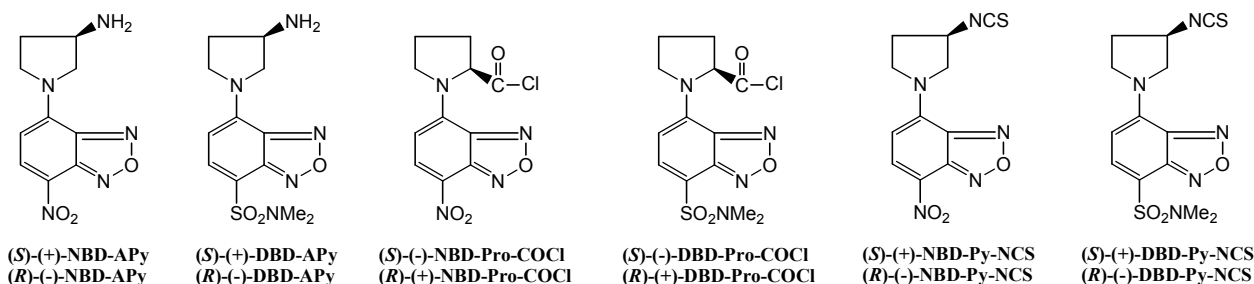
DBD-PZ

DBD-ED

NBD-CO-Hz

### Measurement of Optical Purity:

NBD-,DBD-APy<sup>18,19</sup>, NBD-,DBD-Pro-COCl<sup>20,21</sup>, and NBD-,DBD-Py-NCS<sup>22-25</sup> have a chiral center in the pyrrolidine moiety which is introduced at the 4-(7-) position of the NBD-, DBD- structure, respectively. NBD-, DBD-APy reacts with carboxylic acids, NBD-, DBD-Pro-COCl with alcohols, amines, NBD-Py-NCS with amines, and DBD-Py-NCS with thiols, amines, respectively, to give the corresponding diastereomers which are extremely well separated over HPLC using achiral column. Furthermore, application of NBD-, DBD-Py-NCS to the Edman degradation has been reported.



(S)-(+)-NBD-APy  
(R)-(-)-NBD-APy

(S)-(+)-DBD-APy  
(R)-(-)-DBD-APy

(S)-(-)-NBD-Pro-COCl  
(R)-(+)-NBD-Pro-COCl

(S)-(-)-DBD-Pro-COCl  
(R)-(+)-DBD-Pro-COCl

(S)-(+)-NBD-Py-NCS  
(R)-(-)-NBD-Py-NCS

(S)-(+)-DBD-Py-NCS  
(R)-(-)-DBD-Py-NCS

### High Sensitivity:

The labeled targets with NBD- and DBD- derivatives fluoresce strongly and therefore are detected with high sensitivity. They have long wavelength fluorescence so the analyses will not be affected by interference from contamination. For higher sensitivity, chemiluminescence<sup>26,27</sup> or laser fluorescence detection can be employed.

### Products

A5592	NBD-Cl	5g	1g	A5562	(S)-(+)-NBD-APy	100mg
A5593	NBD-F		100mg	A5563	(R)-(-)-NBD-APy	100mg
A5595	DBD-F		100mg	A5560	(S)-(+)-DBD-APy	100mg
A5575	DBD-NCS		100mg	A5561	(R)-(-)-DBD-APy	100mg
A5557	NBD-H		100mg	A5567	(S)-(-)-NBD-Pro-COCl	100mg
A5556	DBD-H		100mg	A5566	(R)-(+)-NBD-Pro-COCl	100mg
A5554	NBD-PZ		100mg	A5564	(S)-(-)-DBD-Pro-COCl	100mg
A5555	DBD-PZ		100mg	A5565	(R)-(+)-DBD-Pro-COCl	100mg
A5572	NBD-COCl		100mg	A5578	(S)-(+)-NBD-Py-NCS	100mg
A5558	DBD-COCl		100mg	A5577	(R)-(-)-NBD-Py-NCS	100mg
A5573	NBD-CO-Hz		100mg	A5569	(S)-(+)-DBD-Py-NCS	100mg
A5571	DBD-CO-Hz		100mg	A5568	(R)-(-)-DBD-Py-NCS	100mg
A5574	DBD-ED		100mg	A5596	DAABD-Cl	100mg

### References

- 1) K. Imai, Y. Watanabe, *Anal. Chim. Acta*, **1981**, 130, 377.
- 2) Y. Watanabe, K. Imai, *J. Chromatogr.*, **1982**, 239, 723.
- 3) Y. Watanabe, K. Imai, *J. Chromatogr.*, **1984**, 309, 279.
- 4) T. Toyo'oka, T. Suzuki, Y. Saito, S. Uzu, K. Imai, *Analyst*, **1989**, 114, 413.
- 5) T. Toyo'oka, T. Suzuki, Y. Saito, S. Uzu, K. Imai, *Analyst*, **1989**, 114, 1233.
- 6) K. Imai, S. Uzu, T. Toyo'oka, *J. Pharm. Biomed. Anal.*, **1989**, 7, 1395.
- 7) Y. Huang, H. Matsunaga, A. Toriba, T. Santa, T. Fukushima, K. Imai, *Anal. Biochem.*, **1999**, 270, 257.
- 8) H. Matsunaga, T. Santa, K. Hagiwara, H. Homma, K. Imai, S. Uzu, K. Nakashima, S. Akiyama, *Anal. Chem.*, **1995**, 67, 4276.
- 9) K. Imai, S. Uzu, K. Nakashima, S. Akiyama, *Biomed. Chromatogr.*, **1993**, 7, 56.
- 10) M. Masuda, C. Toriumi, T. Santa, K. Imai, *Anal. Chem.*, **2004**, 76, 728; M. Masuda, H. Saimaru, N. Takamura, K. Imai, *Biomed. Chromatogr.*, **2005**, 19, 556.
- 11) K. Imai, T. Fukushima, H. Yokosu, *Biomed. Chromatogr.*, **1994**, 8, 107.
- 12) Tokyo Kasei Kogyo, Jpn. Kokai Tokkyo Koho H07-238075, **1995**.
- 13) S. Uzu, S. Kanda, K. Imai, K. Nakashima, S. Akiyama, *Analyst*, **1990**, 115, 1477.
- 14) T. Toyo'oka, M. Ishibashi, Y. Takeda, K. Nakashima, S. Akiyama, S. Uzu, K. Imai, *J. Chromatogr.*, **1991**, 588, 61.
- 15) Tokyo Kasei Kogyo, Jpn. Kokai Tokkyo Koho H10-218871, **1998**.
- 16) P. Prados, T. Fukushima, T. Santa, H. Homma, M. Tsunoda, S. Al-Kindy, S. Mori, H. Yokosu, K. Imai, *Anal. Chim. Acta*, **1997**, 344, 227.
- 17) T. Santa, A. Takeda, S. Uchiyama, T. Fukushima, H. Homma, S. Suzuki, H. Yokosu, C. K. Lim, K. Imai, *J. Pharm. Biomed. Anal.*, **1998**, 17, 1065.
- 18) T. Toyo'oka, M. Ishibashi, T. Terao, *Analyst*, **1992**, 117, 727.
- 19) T. Toyo'oka, M. Ishibashi, T. Terao, *J. Chromatogr.*, **1992**, 625, 357.
- 20) Tokyo Kasei Kogyo, Jpn. Kokai Tokkyo Koho H06-184141, **1994**.
- 21) Tokyo Kasei Kogyo, Jpn. Kokai Tokkyo Koho H07-188224, **1995**.
- 22) T. Toyo'oka, Y-M. Liu, *Analyst*, **1995**, 120, 385.
- 23) T. Toyo'oka, Y-M. Liu, *J. Chromatogr. A*, **1995**, 689, 23.
- 24) T. Toyo'oka, Y-M. Liu, *Chromatographia*, **1995**, 40, 645.
- 25) Y-M. Liu, J-R. Miao, T. Toyo'oka, *Anal. Chim. Acta*, **1995**, 314, 169.
- 26) S. Uzu, K. Imai, K. Nakashima, S. Akiyama, *Biomed. Chromatogr.*, **1991**, 5, 184.
- 27) S. Uzu, K. Imai, K. Nakashima, S. Akiyama, *Analyst*, **1991**, 116, 1353.