HPLC Labeling Reagents

HPLC is utilized extensively as a means of detecting and determining trace components. Labeling objective substances for analysis with labeling reagents appropriate for detection methods has been performed in order to obtain higher sensitivity and selectivity. Many labeling reagents have been reported for this purpose. We picked up a part of them and sell them as our TCI-Ace series.

All HPLC labeling reagents are high quality products, so you can make use of these products to achieve high quality analyses.

---- Products List by detection and functional groups ----

### UV Detection

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see also TCI product number list (p.99)
Labeling Reagent for UV Detection of Carboxylic Acids

4-Bromophenacyl Bromide 5g [A5501]

The compound A5501 is an HPLC labeling reagent, which has a bromoacetyl group and easily reacts with a carboxyl group to form the corresponding ester in the presence of a base. The resultant ester is stable and can reach the detector without any decomposition under reversed phase HPLC. An excellent chromatogram can be obtained by measuring at 254 nm, an absorption wavelength that is generally used for UV detection.

![Chemical Structure](image)

Application examples:

[Fatty acids] Dissolve a sample in methanol or water, and then neutralize the sample solution with methanol solution of KOH-crown ether. Evaporate to dryness under reduced pressure, and then you will see a generally almost white solid substance remaining (potassium salt of fatty acid). Next, add the HPLC labeling reagent A5501 with acetonitrile solution* of 18-crown 6-ether to this white solid and further add acetonitrile for a volume up to 10 mL. Incubate the solution at 80 ºC for 15 min. Cool the resultant solution to room temperature and use it as an HPLC sample.

* Benzene can be used in the place of acetonitrile. The mixing ratio (molar ratio) for the HPLC labeling reagent A5501 and 18-crown 6-ether should be 20 to 1 and 10 to 1 for the sample fatty acid concentrations at 0.5~20 mM and less than 0.5 mM, respectively. Use the excessive amount of the reagent A5501.

[Others] Dicarboxyl acids, synthetic prostaglandins, unsaturated fatty acids, alkyl methylphosphonate, ganglioside, betaine

References

Chromatogram of fatty acids as 4-bromophenacyl esters

Column : Kaseisorb LC C\textsubscript{1}-60-5
Column Size : 4.6 mm I.D. × 150 mm
Mobile Phase : CH\textsubscript{3}CN / H\textsubscript{2}O = 80 / 20
Detection : UV 254 nm
Flow Rate : 1 mL / min

Sample :
1. Lauric Acid
2. Myristic Acid
3. Palmitic Acid
4. Stearic Acid
5. Arachidic Acid
Labeling Reagent for UV/Fluorescence Detection
of Carboxylic Acids

9-Chloromethylanthracene

The compound A5502, an HPLC labeling reagent which has a chloromethyl group, easily reacts with a carboxyl group to form the corresponding ester in the presence of a base. The resultant ester is stable and can reach the detector without any decomposition under reversed phase HPLC. An excellent chromatogram can be obtained by measuring at 254 nm, an absorption wavelength that is generally used for UV detection. Furthermore, it has a characteristic fluorescence based on an anthracene skeleton, thus carboxylic acids can be detected with the detection limit of 2 fmol by fluorescence detection analysis at the excitation and emission wavelengths of 365 nm and 412 nm, respectively.

Application example:
[Fatty acids]

Dissolve 60 μg of a sample in 1 mL of DMF, and add 1 mL of tetramethylammonium hydroxide / DMF solution (1 x 10⁻³ M) and 1 mL of the labeling reagent A5502 / cyclohexane solution (5 x 10⁻³ M). Close the cap of the reaction vessel and incubate the solution at 75 °C for 30 min. Cool the resultant solution to room temperature and use it as an HPLC sample.

The detection limit = 0.1 pmol (UV detection: 254 nm)
The detection limit = 2 fmol (Fluorescence detection: λex 365 nm, λem 412 nm)

Reference
Chromatogram of fatty acids as 9-anthrylmethyl esters

Column : Kaseisorb LC C8-60-5
Column Size : 4.6 mm I.D. × 150 mm
Mobile Phase : CH3CN / H2O = 90 / 10
Detection : UV 254 nm
Flow Rate : 1 mL / min
Sample : 1. Lauric Acid
2. Tridecanoic Acid
3. Myristic Acid
4. Pentadecanoic Acid
5. Palmitic Acid

Column : Kaseisorb LC C8-60-5
Column Size : 4.6 mm I.D. × 150 mm
Mobile Phase : CH3CN / H2O = 90 / 10
Detection : Fluorescence λex 365 nm λem 412 nm
Flow Rate : 1 mL / min
Sample : 1. Lauric Acid
2. Tridecanoic Acid
3. Myristic Acid
4. Pentadecanoic Acid
5. Palmitic Acid
Labeling Reagent for UV Detection
of Carboxylic Acids

*N-Chloromethyl-4-nitrophthalimide*  1g  5g [A5503]

The compound **A5503** is an HPLC labeling reagent, which has a chloromethyl group and easily reacts with a carboxyl group to form the corresponding ester in the presence of a base. The resultant ester is stable and can reach the detector without any decomposition under reversed phase HPLC. An excellent chromatogram can be obtained by measuring at 230 nm.

**Application example:**

[Fatty acids] ¹, ²

Dissolve 3 mg of a sample in 1 mL of acetonitrile, and add 1 mL of the labeling reagent **A5503** / acetonitrile solution (11 mg / mL) and 1 mL of triethylamine / acetonitrile solution (5 mg / mL). Close the cap of the reaction vessel and incubate the solution at 60 °C for 1 h. Cool the resultant solution to room temperature and use it as an HPLC sample. In the case of using alkali metal salts and crown ethers, the esterification reaction is completed in 15 min at 60 °C. Cool the resultant solution to room temperature and use it as an HPLC sample.

**References**

Chromatogram of fatty acids as (4-nitrophthalimido)methyl esters

Column : Kaseisorb LC ODS-300-5  
Column Size : 4.6 mml.D. × 150 mm  
Mobile Phase : CH₃CN / H₂O = 85 / 15  
Detection : UV 230 nm  
Flow Rate : 1 mL / min  
Sample : 1. Pentadecanoic Acid  
2. Palmitic Acid  
3. Margaric Acid
Labeling Reagent for UV Detection
of Carboxylic Acids

*N*-Chloromethylphthalimide 5g [A5504]

The compound A5504 is an HPLC labeling reagent, which has a chloromethyl group and easily reacts with a carboxyl group to form an ester in the presence of a base. The resultant ester is stable and can reach the detector without any decomposition under reversed phase HPLC. An excellent chromatogram can be obtained by measuring at 254 nm, an absorption wavelength that is generally used for UV detection.

**Application example:**

[Fatty acids] ¹

Dissolve 3 mg of a sample in 1 mL of acetonitrile, and add 1 mL of the labeling reagent A5504/ acetonitrile solution (10 mg / mL) and 1 mL of triethylamine / acetonitrile solution (5 mg / mL). Close the cap of the reaction vessel and incubate the solution at 60 °C for 1 h. Cool the resultant solution to room temperature and use it as an HPLC sample. In the case of using alkali metal salts and crown ethers, the esterification reaction is completed in 5 min at 60 °C. Cool the resultant solution to room temperature and use it as an HPLC sample.

**Reference**

Chromatogram of fatty acids as phthalimidomethyl esters

Column: Kaseisorb LC C₈-60-5
Column Size: 4.6 mm I.D. × 150 mm
Mobile Phase: CH₃CN / H₂O =
70 / 30 → 100 / 0
20 min linear gradient
Detection: UV 254 nm
Flow Rate: 1 mL / min
Sample:
1. Caproic Acid
2. Caprylic Acid
3. Capric Acid
4. Undecanoic Acid
5. Lauric Acid
6. Tridecanoic Acid
7. Myristic Acid
8. Palmitic Acid
9. Stearic Acid
10. Arachidic Acid
Labeling Reagent for UV Detection
of Carboxylic Acids

3'-Methoxyphenacyl Bromide

The compound A5505 is an HPLC labeling reagent, which has a bromoacetyl group and easily reacts with a carboxyl group to form the corresponding ester in the presence of a base. The resultant ester is stable and can reach the detector without any decomposition under reversed phase HPLC. An excellent chromatogram can be obtained by measuring at 254 nm, an absorption wavelength that is generally used for UV detection.

Application example:
[Fatty acids] 1~3) Dissolve 4 mg of a sample in 1 mL of N,N-dimethylformamide (DMF), and add the labeling reagent A5505 (10 mg) in DMF (1 mL) and N,N-diisopropylethylamine (10 mg) in DMF (2 mL). Close the cap of the reaction vessel and incubate the solution at 60 °C for 1 h. Cool the resultant solution to room temperature and use it as an HPLC sample.

References
2) N. E. Bussell, R. A. Miller, J. Liquid Chromatogr. 1979, 2, 697.
3) N. E. Bussell, A. Gross, R. A. Miller, J. Liquid Chromatogr. 1979, 2, 1337.
Chromatogram of fatty acids as 3'-methoxyphenacyl esters

Column: Kaseisorb LC C\textsubscript{8}-60-5
Column Size: 4.6 mm I.D. × 150 mm
Mobile Phase: CH\textsubscript{3}CN / H\textsubscript{2}O = 90 / 10
Detection: UV 254 nm
Flow Rate: 1 mL / min
Sample: 1. Linolenic Acid
       2. Linolic Acid
       3. Oleic Acid
       4. Stearic Acid
Labeling Reagent for UV Detection
of Carboxylic Acids

\[ O-(4-Nitrobenzyl)-N,N'-diisopropylisourea \]

The compound A5506 easily reacts with a carboxyl group to form the corresponding ester without using a catalyst or an activating agent. The resultant ester is stable and can reach the detector without any decomposition under reversed phase HPLC. An excellent chromatogram can be obtained by measuring at 254 nm, an absorption wavelength that is generally used for UV detection.

Application example:

[Fatty acids] ¹

Dissolve 5 mg of a sample in CH₂Cl₂ (1 mL), and add the labeling reagent A5506 (20 mg) in CH₂Cl₂ (2 mL). Close the cap of the reaction vessel and incubate the solution at 80 ºC for 2 h. Cool the resultant solution to room temperature and use it as an HPLC sample.

References

Chromatogram of fatty acids as 4-nitrobenzyl esters

Column: Kaseisorb LC C_1-60-5
Column Size: 4.6 mm I.D. × 150 mm
Mobile Phase: CH_3CN / H_2O = 75 / 25
Detection: UV 254 nm
Flow Rate: 1 mL / min
Sample: 1. Linolenic Acid
2. Linolic Acid
3. Oleic Acid
4. Stearic Acid
Labeling Reagent for UV Detection of Carboxylic Acids

Phenacyl Bromide

5g [A5508]

The compound A5508 is an HPLC labeling reagent, which has a bromoacetyl group and easily reacts with a carboxyl group to form the corresponding ester in the presence of a base. The resultant ester is stable and can reach the detector without any decomposition under reversed phase HPLC. An excellent chromatogram can be obtained by measuring at 254 nm, an absorption wavelength that is generally used for UV detection.

\[
\begin{array}{c}
\text{R-COH} \xrightarrow{\text{[C(C_2H_5)_3N]} \text{Acetone, 50 °C}} \text{R-COCH}_2\text{C} \\text{[A5508]} \\
\end{array}
\]

Application examples:

[Fatty acids] ¹) Mix ca. 100 μg of a sample, 10 μL of the labeling reagent A5508 in acetone (12 mg / mL) and 10 μL of triethylamine in acetone (10 mg / mL), and incubate the solution at 50 °C for 2 h. Cool the resultant solution to room temperature and use it as an HPLC sample.

[Others]
Bile acids²), fatty acids³), carboxylic acids in wine⁴)

References
Chromatogram of fatty acids as phenacyl esters

- Column: Kaseisorb LC ODS-100-5
- Column Size: 4.6 mm I.D. × 150 mm
- Mobile Phase: CH₃CN / H₂O = 90 / 10
- Detection: UV 254 nm
- Flow Rate: 1 mL / min
- Sample:
  1. Caproic Acid
  2. Caprylic Acid
  3. Capric Acid
  4. Undecanoic Acid
  5. Lauric Acid
  6. Myristic Acid
Labeling Reagent for UV Detection
of Alcohols and Amines

3,5-Dinitrobenzoyl Chloride

The compound A5511 is an HPLC labeling reagent, which easily reacts with a hydroxyl group or an amino group to form the corresponding ester or amide, respectively. The resultant ester or amide is stable and can reach the detector without any decomposition under reversed phase HPLC. An excellent chromatogram can be obtained by measuring at 254 nm, an absorption wavelength that is generally used for UV detection.

Application examples:

[Alcohols] 1)
Dissolve 1~5 mg of a sample in 5 mL of THF, and add 40 mg of the labeling reagent A5511 and a few drops of pyridine. Close the cap of the reaction vessel and incubate the solution at 60 °C for 1 h. Cool the resultant solution to room temperature and use it as an HPLC sample.

Clean up before injection is recommended when pyridine or triethylamine is added to trap generated HCl. Generally, evaporate the solvent, extract with ether and wash the ether layer with diluted hydrochloric acid and water.

[Others]
Analysis of mono- and diethylene glycols in polyethylene glycol2), aliphatic alcohols3)

References
Chromatogram of alcohols as 3,5-dinitrobenzoic acid esters

Column : Kaseisorb LC ODS-300-5
Column Size : 4.6 mm I.D. × 150 mm
Mobile Phase : CH₃CN / H₂O = 55 / 45
Detection : UV 254 nm
Flow Rate : 1 mL / min
Sample : 1. Ethylene glycol
         2. Ethanol
         3. Propanol
         4. Butanol

Time, min
0 10 20
Labeling Reagent for UV Detection
of Amines

2,4-Dinitrofluorobenzene

The compound **A5512** easily reacts with an amino group to form the corresponding 2,4-dinitrophenylamine derivative. The resultant derivative is stable and can reach the detector without any decomposition under reversed phase HPLC. An excellent chromatogram can be obtained by measuring at 254 nm, an absorption wavelength that is generally used for UV detection.

Application examples:

[Amines]
A sample (free amine) 10 mg, chloroform 1 mL, and labeling reagent **A5512** (10 eq. excess amount of the sample) are mixed, and incubated at 60 °C for 1 h. After cooling to room temperature, use it as an HPLC sample. **A5512** is also used for derivatization of amino acids.  

[Others]
Aminoglycosides

References
Chromatogram of alkylamines as 2,4-dinitrophenyl derivatives

Column: Kaseisorb LC C4-60-5
Column Size: 4.6 mm I.D. × 150 mm
Mobile Phase: CH₃CN / H₂O = 45 / 55
Detection: UV 254 nm
Flow Rate: 1 mL / min
Sample:
1. Labeling Reagent
2. Diethylamine
3. Propylamine
Labeling Reagent for UV Detection of Amines
Phenyl Isothiocyanate

The compound A5513 is an HPLC labeling reagent, which has an isothiocyano group, can easily react with an amino group to form the corresponding thiourea. The resultant thiourea can be also derivatized into a phenylthiohydantoin (PTH) derivative under acidic conditions. The PTH is stable enough to reach the detector without any decomposition under reversed phase HPLC. An excellent chromatogram can be obtained by measuring at 269 nm for UV detection.

Application example:
[Amino acids, Peptides]
1.5 μmol of a sample is dissolved into 1 mL of 60% aqueous pyridine solution containing labeling reagent A5513 (15 mg), and incubated at 40 °C for 1 h. After cooling to room temperature, the reaction mixture is diluted with 1 mL of water, and excess amount of A5513 is removed by extraction (benzene 2 mL x 4 times). The aqueous layer is evaporated, and dried in desiccator. To the residue, 1.5 mL of mixed solution (3 N HCl and 60% AcOH, 1 : 1) is added to hydrolyzed at 40 °C for 30 min under a nitrogen atmosphere. After cooling to room temperature, the reaction mixture is diluted with 2 mL of water, and extracted with 2 mL of ethyl acetate, next 2 mL of benzene. The organic layers are combined to use it as an HPLC sample.

References
Chromatogram of amino acids as PTH derivatives

Column : Kaseisorb LC C8-60-5
Column Size : 4.6 mm I.D. × 300 mm
Mobile Phase : A ; CH3CN
               : B ; 40 mM CH3COONa
               : C ; H2O
Detection : UV 269 nm
Flow Rate : 1 mL / min

Time(min) A(%)  B(%)  C(%) 
0   36 20 44  
3   42 20 38  
4   45 25 30  
5   50 30 20  
9   52 30 18  
12  65  5 30  
13  36 20 44  
(temp.40 ºC)

Labeling Reagent for UV Detection
of Chiral Amines

2,3,4,6-Tetra-O-acetyl-β-D-glucopyranosyl Isothiocyanate (=GITC) 100mg 1g [A5514]

The compound A5514 is an HPLC labeling reagent for optical purity determination, which has a glyco-moiety and an isothiocyano group, and easily reacts with an amino group to form the corresponding thiourea. The resultant thiourea is stable enough to reach the detector without any decomposition under reversed phase HPLC. An excellent chromatogram can be obtained by measuring at 254 nm, an absorption wavelength that is generally used for UV detection. Furthermore, A5514 reacts with a racemic amine to generate diastereomers, which can be efficiently separated by reversed phase HPLC.

Application examples:
[Amino acids]
1) 5 mg of an amino acid is dissolved in 50% (V/V) aqueous acetonitrile containing 0.4% (W/V) triethylamine in order to give a final volume of 10 mL. To 50 μL of this solution 50 μL of 0.2% (W/V) labeling reagent A5514 in acetonitrile are added. The resulting mixture is shaken at room temperature for 30 min and used as an HPLC sample.

[Others]
Propranolol[1], trimetopin[1]

References
Chromatogram of thiourea derivatives formed from amino acids with GITC

Column : Kaseisorb LC ODS Super
Column Size : 4.6 mm I.D. × 150 mm
Mobile Phase : 10 mM Phosphate buffer / Methanol
               = 45 / 55 (pH 3.0)
Detection : UV 254 nm
Temperature : 25 ºC
Flow Rate : 1 mL / min
Sample : 1. Aspartic Acid
         2. L-Valine
         3. D-Valine
         4. L-Tryptophan
         5. D-Tryptophan
Labeling Reagent for UV Detection

of Chiral Amines

2,3,4,6-Tetra-O-benzoyl-β-D-glucopyranosyl Isothiocyanate (=BGIT) 100mg 1g [A5515]

The compound A5515 is an HPLC labeling reagent for optical purity determination, which has a glyco-moiety and an isothiocyano group, and easily reacts with an amino group to form the corresponding thiourea. The resultant thiourea is stable and can reach the detector without any decomposition under reversed phase HPLC. An excellent chromatogram can be obtained by measuring at 254 nm, an absorption wavelength that is generally used for UV detection. Furthermore, A5515 reacts with a racemic amine to generate diastereomers, which can be efficiently separated by reversed phase HPLC.

Application example:
[Amino acids]

5 mg of an amino acid is dissolved in 50% (V/V) aqueous acetonitrile containing 0.55% (V/V) triethylamine in order to give a final volume of 10 mL. To 50 μL of this solution 50 μL of 0.66% (W/V) labeling reagent A5515 in acetonitrile are added. The resulting mixture is shaken at room temperature for 30 min, then 10 μL of 0.26% (V/V) ethanolamine in acetonitrile are added and shaken for another 10 min. The mixture is diluted with acetonitrile to a final volume of 1 mL and used as an HPLC sample.

Reference
Chromatogram of thiourea derivatives formed from amino acids with BGIT

Column: Kaseisorb LC ODS Super
Column Size: 4.6 mm I.D. × 150 mm
Mobile Phase: 10 mM Phosphate buffer / CH₃CN = 35 / 65 (pH 3.0)
Detection: UV 254 nm
Temperature: 25 ºC
Flow Rate: 1 mL / min
Sample: 1. L-Phenylalanine
        2. D-Phenylalanine
Labeling Reagent for UV Detection
of Amines

*N-Succinimidyl 4-Nitrophenylacetate*  
1g [A5522]

The compound **A5522** is an HPLC labeling reagent, which has a succinimidyl group, which can easily react with an amino group to form the corresponding amide derivative. The resultant amide is stable enough to reach the detector without any decomposition under reversed phase HPLC. An excellent chromatogram can be obtained by measuring at 254 nm, an absorption wavelength that is generally used for UV detection.

[Chemical structure image]

**Application examples:**

**[Alkylamines]**

1~5 mg of a sample (free amine), 5 mL of THF, and 50 mg of labeling reagent **A5522** are mixed, and incubated at 60°C for 1 h. After cooling to room temperature, use it as an HPLC sample. If it is necessary to remove the unreacted labeling reagent and by-product, *N*-hydroxysuccinimide, evaporate the solvent at a low temperature under a nitrogen atmosphere. Dissolve the residue in 2~3 mL of ether and wash with aqueous NaHCO₃ and water.

**[Others]**

Drugs (amphetamine, methamphetamine)¹

**Reference**

Chromatogram of alkylamines as 4-nitrophenylacetamides

Column: Kaseisorb LC ODS-100-5
Column Size: 4.6 mm I.D. × 150 mm
Mobile Phase: CH₃OH / H₂O = 60 / 40
Detection: UV 254 nm
Flow Rate: 1 mL/min
Sample:
1. Propylamine
2. Diethylamine
3. Butylamine
4. Ethylpropylamine
5. Isoamylamine
6. Amylamine
7. Dipropylamine
8. Hexylamine
Labeling Reagents for UV Detection of Chiral Amines

$N^\alpha$-(5-Fluoro-2,4-dinitrophenyl)-L-leucinamide 100mg [A5523]
$N^\alpha$-(5-Fluoro-2,4-dinitrophenyl)-D-leucinamide 100mg 1g [A5524]

The compounds A5523 and A5524 are HPLC labeling reagents for optical purity determination, and can easily react with amino groups. A5523 or A5524 reacts with a racemic amino acid to generate diastereomers, which can be efficiently separated by reversed phase HPLC. The absolute configuration of amino acids also can be non-empirically determined with use of A5523 and A5524. Furthermore, high sensitive analyses can easily be accomplished using LC-MS. [The detection limit: 5 pmol (ESI LC-MS)]

Example: L-form

Application example:
[Amino acids] 2)
To 50 μL of a 50 mM aqueous solution of amino acids are added 20 μL of 1 M NaHCO$_3$ and then 100 μL of 1% labeling reagent A5523 or A5524 in acetone. The solution is incubated at 37 °C for 1 h. Reactions are quenched by addition of 20 μL of 1 N HCl. Samples are diluted with 810 μL of acetonitrile, and 1 μL of this solution is analyzed by LC-MS.

References
Chromatogram of amino acids as L-FDLA derivatives

Column: Kaseisorb LC ODS 2000
Column Size: 2.0 mmI.D. × 150 mm
Mobile Phase: A- 20 mM Ammonium Acetate (pH 4)
B- Methanol

<table>
<thead>
<tr>
<th>Time(min)</th>
<th>A(%)</th>
<th>B(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>90</td>
<td>10</td>
</tr>
<tr>
<td>4</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>20</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>23</td>
<td>0</td>
<td>100</td>
</tr>
</tbody>
</table>

Temperature: 40 ºC
Flow Rate: 0.2 mL / min
Instrument: Hitachi M-8000 LC/3DQ MS
Ionization Method: ESI-AD

1. L-Leucine
2. D-Leucine
3. L-Alanine
4. D-Alanine
5. L-Phenylalanine
6. D-Phenylalanine
Labeling Reagent for UV Detection
of Carbonyl Compounds

2,4-Dinitrophenylhydrazine Hydrochloride

5g [A5531]

The compound A5531 is an HPLC labeling reagent, which has a hydrazino group and easily reacts with a carbonyl group to form the corresponding hydrazones. The resultant hydrazone is stable enough to reach the detector without any decomposition under reversed phase HPLC. An excellent chromatogram can be obtained by measuring at 254 nm, an absorption wavelength that is generally used for UV detection.

Application examples:

[Aldehydes]
1 mg of a sample, 1 mg of the labeling reagent A5531, 1 mL of methanol, and 0.5 mL of 1 N HCl are mixed. Close the cap of the reaction vessel and incubate the mixture at 40 ºC for 10 min. After cooling to room temperature, use it as the HPLC sample solution.

[Keto acids] 1,2)
A sample is dissolved in 1 mL of diluted HCl solution containing labeling reagent A5531 (500 μmol / 2 N HCl 100 mL). Incubate the mixture at 30 ºC for 30 min. (The reactions are completed in 5 min and 20 min for ketomonocarboxylic acids and ketodicarboxylic acids, respectively.) It is preferable to add over 4 eq. amount of the labeling reagent, and resultant hydrazones can be extracted with ethyl acetate.

[Urine, 17-Ketosteroids in blood plasma] 3,4)
A sample is dissolved into methanol, and acidified with 3~4 drops of conc. HCl. Excess amount of 0.2% labeling reagent A5531 in methanol is added. Incubate the mixture at 50 ºC for 5 min.

[Others]
Aliphatic carbonyl compounds 5,6), aliphatic aldehydes 7~9)

References

AZ-532
Chromatogram of aldehydes as 2,4-dinitrophenylhydrazone

Column: Kaseisorb LC ODS-60-5
Column Size: 4.6 mm I.D. × 150 mm
Mobile Phase: CH₃CN / H₂O = 70 / 30
Detection: UV 254 nm
Flow Rate: 1 mL / min
Sample:
1. Formaldehyde
2. Acetaldehyde
3. Propionaldehyde
4. Butyraldehyde
5. Valeraldehyde
6. Capronaldehyde
7. Heptylaldehyde

9) M. Uehori, K. Kuwata, Y. Yamazaki, Annual report of Environmental Pollution Control Center Osaka Prefecture 1982, 5, 27.
Labeling Reagent for UV Detection
of Carbonyl Compounds

**O-4-Nitrobenzylhydroxylamine Hydrochloride**

![Chemical Structure](image)

The compound **A5532** is an HPLC labeling reagent, which has a hydroxylamino moiety, can easily react with a carbonyl group to form the corresponding oxime. The resultant oxime is stable enough to reach the detector without any decomposition under reversed phase HPLC. An excellent chromatogram can be obtained by measuring at 254 nm, an absorption wavelength that is generally used for UV detection.

![Chemical Reaction](image)

**Application example:**

**[Aldehydes]**

1~5 mg of a sample, 4 mL of methanol, 2 drops of triethylamine, and 40 mg of the labeling reagent **A5532** are mixed. Close the cap of the reaction vessel and incubate the mixture at 65 °C for 1 h. After cooling to room temperature, use it as the HPLC sample solution. If it is necessary to remove the unreacted labeling reagent and triethylamine, evaporate the solvent at a low temperature under a nitrogen atmosphere. Dissolve the residue in 2~3 mL of ether and wash with dilute HCl and water.

**Reference**

Chromatogram of aldehydes as 4-nitrobenzyloximes

Column: Kaseisorb LC ODS-300-5
Column Size: 4.6 mm I.D. × 150 mm
Mobile Phase: CH$_3$CN / H$_2$O = 60 / 40
Detection: UV 254 nm
Flow Rate: 1 mL / min
Sample: 1. Propionaldehyde
       2. Butyraldehyde
       3. Valeraldehyde
       4. Capronaldehyde
Labeling Reagent for Fluorescence Detection of Carboxylic Acids

**Br-Mmc (=4-Bromomethyl-7-methoxycoumarin)**

The compound A5551 is an HPLC fluorescence labeling reagent, which has a bromomethyl group, can easily react with a carboxyl group to form the corresponding ester in the presence of a base. The resultant ester is stable enough to reach the detector without any decomposition under reversed phase HPLC. Furthermore, it has a characteristic fluorescence based on a coumarin skeleton, thus an excellent chromatogram can be obtained by fluorescence detection at the excitation and emission wavelengths of 328 nm and 380 nm, respectively.

**Application examples:**

[Fatty acids] ¹

0.05 g of the labeling reagent A5551 and 0.5 g of K₂CO₃ powder is added to a acetone solution (5 mL) of a sample (0.01 g), and incubate at 60 °C for 1 h. After cooling to room temperature, use it as the HPLC sample solution.

[Others]

Carboxylic acids, aliphatic acids, dicarboxylic acids, prostaglandins, bile acids, barbitals

**References**

Chromatogram of fatty acids as methoxycoumarinylmethyl esters

Column: Kaseisorb LC ODS-100-5
Column Size: 4.6 mm I.D. x 150 mm
Mobile Phase: CH₃CN / H₂O = 85 / 15
Detection: Fluorescence λₑₓ 328 nm, λₑₘ 380 nm
Flow Rate: 1 mL / min
Sample:
1. Pelargonic Acid
2. Capric Acid
3. Undecanoic Acid
Labeling Reagent for Fluorescence Detection of Carbonyl Compounds

Dansyl Hydrazine

The compound A5552 is an HPLC fluorescence labeling reagent, and can easily react with a carbonyl group to form the corresponding hydrazone. The resultant hydrazone is stable enough to reach the detector without any decomposition under reversed phase HPLC. An excellent chromatogram can be obtained by fluorescence detection at the excitation and emission wavelengths of 340 nm and 525 nm, respectively.

Application examples:

[Ketosteroids] 1-4)
A dried sample, 0.2 mL of an alcoholic hydrochloric acid (conc. HCl 0.65 mL / ethanol 1 L), and 0.2 mL of the labeling reagent A5552 in alcohol (2 mg / mL) are mixed, and heated on a water bath for 10 min. 0.2 mL of alcohol containing sodium pyruvate (5 mg / mL) is added to decompose the excess labeling reagent. The reaction mixture is allowed to stand at room temperature for 15 min, ether (6 mL) and 0.5 N NaOH (3 mL) are added and shaken. After an extraction procedure, the solvent is evaporated, chloroform (0.2~0.5 mL) is added to the residue, and use as the HPLC sample.

[Others]
Hydrocortisone in body fluid 3,4), reducing sugars, steroids in serum and urine 5

References
Chromatogram of aldehydes as dansyl hydrazones

Column: Kaseisorb LC ODS-100-5
Column Size: 4.6 mm I.D. × 150 mm
Mobile Phase: CH₃CN / H₂O = 65 / 35
Detection: Fluorescence λ<sub>ex</sub> 340 nm
          λ<sub>em</sub> 525 nm
Flow Rate: 1 mL / min
Sample: 1. Valeraldehyde
       2. Capronaldehyde
       3. Enanthic Aldehyde
Labeling Reagent for Fluorescence Detection of Carboxylic Acids

3-Bromomethyl-7-methoxy-1,4-benzoxazin-2-one

The compound A5553 is an HPLC fluorescence labeling reagent, which has a bromomethyl group, can easily react with a carboxyl group to form the corresponding ester in the presence of a base. The resultant ester is stable enough to reach the detector without any decomposition under reversed phase HPLC. An excellent chromatogram can be obtained by fluorescence detection at the excitation and emission wavelengths of 355 nm and 430 nm, respectively.

Application example:

[Fatty acids] 1) A solution of the labeling reagent A5553 (0.1 mL, 1.0 mM acetonitrile solution) is added to a solution of a fatty acid (0.5 mL, 0.2~10 nmol in acetonitrile). To this solution, a saturated K₂CO₃ / acetonitrile solution (0.5 mL) containing 18-crown 6-ether (5.7 mM) is added, and incubate at 40 °C for 30 min. After cooling to room temperature, use it as the HPLC sample solution.

References

2) A. Nakanishi, H. Naganuma, J. Kondo, K. Watanabe, Y. Kawahara, Program and Abstracts 109th Congress of the Pharmaceutical Society of Japan, 6TA, 2-1.
Chromatogram of fatty acids as 7-methoxy-1,4-benzoxazin-2-one-3-methyl ester

Sample: 1. Linolenic Acid
2. Linolic Acid
3. Oleic Acid
4. Stearic Acid

Sample: 1. Butyric Acid (C₄)
2. Valeric Acid (C₅)
3. Caproic Acid (C₆)

Column: Kaseisorb LC ODS-120-5
Column Size: 4.6 mm I.D. × 150 mm
Mobile Phase: CH₃CN / H₂O = 95 / 5
Detection: Fluorescence λₑₓ 355 nm, λₑₘ 430 nm
Temperature: 30 ºC
Flow Rate: 1 mL / min

Column: Kaseisorb LC ODS-120-5
Column Size: 4.6 mm I.D. × 150 mm
Mobile Phase: CH₃CN / H₂O = 50 / 50
Detection: Fluorescence λₑₓ 355 nm, λₑₘ 430 nm
Temperature: 25 ºC
Flow Rate: 1 mL / min
Labeling Reagent for Fluorescence Detection
of Carboxylic Acids

**NBD-PZ (=4-Nitro-7-piperazino-2,1,3-benzoxadiazole)**

100mg [A5554]

The compound **A5554** is an HPLC fluorescence labeling reagent, which has a 2,1,3-benzoxadiazole skeleton and a piperazino group, easily reacts with a carboxyl group at room temperature to form the corresponding amide in the presence of a condensation reagent. An excellent chromatogram can be obtained by fluorescence detection at the excitation and emission wavelengths of 470 nm and 541 nm, respectively. Since their excitation and fluorescence wavelengths are at longer wavelengths, detection has less interference by contaminants. A highly sensitive detection can be done by using laser induced fluorescence detector.

**Application example:**

[Fatty acids]

1) 0.2 mL of 140 mM DEPC or 70 mM 2,2'-dipyridyl disulfide/Ph$_3$P / DMF solution containing a fatty acid (10 μM) is added to 0.2 mL of the labeling reagent **A5554** / DMF or acetonitrile solution (10 mM). React at room temperature for 6 h, then use it as an HPLC sample.

**Reference**

Chromatogram of fatty acids as NBD-PZ derivatives

Column : Kaseisorb LC ODS Super
Column Size : 4.6 mm I.D. × 150 mm
Mobile Phase : CH₃CN
Detection : Fluorescence λex 470 nm
            λem 541 nm
Temperature : 25 ºC
Flow Rate : 1 mL / min
Sample : 1. Linolenic Acid
        2. Linolic Acid
        3. Oleic Acid
        4. Stearic Acid
Labeling Reagent for Fluorescence Detection of Carboxylic Acids

DBD-PZ
[=4-(N,N-Dimethylaminosulfonyl)-7-piperazino-2,1,3-benzoxadiazole]

The compound A5555 is an HPLC fluorescence labeling reagent, which has a 2,1,3-benzoxadiazole skeleton and a piperazino group, easily reacts with a carboxyl group at room temperature to form the corresponding amide in the presence of a condensation reagent. An excellent chromatogram can be obtained by fluorescence detection at the excitation and emission wavelengths of 440 nm and 569 nm, respectively. Since their excitation and fluorescence wavelengths are at longer wavelengths, detection has less interference by contaminants. A highly sensitive analysis can be done by peroxo)xalate chemiluminescence detection.

Application example:
[Fatty acids]
0.2 mL of 140 mM DEPC or 70 mM 2,2'-dipyridyl disulfide-Ph3P / DMF solution containing a fatty acid (10 μM) is added to 0.2 mL of the labeling reagent A5555 / DMF or acetonitrile solution (10 mM). Incubate at room temperature for 6 h, then use it as an HPLC sample.
For example, the detection limit (S/N = 3) for saturated fatty acids (from C13 to C24) is from 3.2 to 4.7 fmol.

References
Chromatogram of fatty acids as DBD-PZ derivatives

Column: Kaseisorb LC ODS Super
Column Size: 4.6 mmI.D. × 150 mm
Detection: Fluorescence $\lambda_{ex}$ 440 nm $\lambda_{em}$ 569 nm
Temperature: 25 °C
Flow Rate: 1 mL / min

Mobile Phase: CH$_3$CN / H$_2$O = 40 / 60
Sample: 1. Acetic Acid
        2. Propionic Acid
        3. Butyric Acid

Mobile Phase: CH$_3$CN
Sample: 1. Linolenic Acid
        2. Linolic Acid
        3. Oleic Acid
        4. Stearic Acid
Labeling Reagent for Fluorescence Detection of Carbonyl Compounds

DBD-H
[=4-(N,N-Dimethylaminosulfonyl)-7-hydrazino-2,1,3-benzoxadiazole]

100mg [A5556]

The compound A5556 is an HPLC fluorescence labeling reagent, which has a 2,1,3-benzoxadiazole skeleton and a hydrazino group, easily reacts with a carbonyl group to form the corresponding hydrazone. The resultant hydrazone is stable enough to reach the detector without any decomposition under reversed phase HPLC. An excellent chromatogram can be obtained by fluorescence detection at the excitation and emission wavelengths of 450 nm and 565 nm, respectively. Since their excitation and fluorescence wavelengths are at longer wavelengths, detection has less interference by contaminants. A highly sensitive detection can be done because of its strong fluorescence.

Application example:
[Aldehydes or ketones]

250 μM of the labeling reagent A5556 and 1.7 μM propionaldehyde are added to acetonitrile containing 0.025% TFA, and reacted at room temperature for 30 min, then use it as the HPLC sample.

For example, the detection limit for propionaldehyde is 120 fmol.

Reference
Chromatogram of aldehyde and ketone as DBD-H derivatives

Column: Kaseisorb LC ODS Super
Column Size: 4.6 mm I.D. × 150 mm
Detection: Fluorescence \( \lambda_{ex} \) 450 nm \( \lambda_{em} \) 565 nm
Temperature: 25 °C
Flow Rate: 1 mL / min

Mobile Phase: \( \text{CH}_3\text{CN} / 0.05\% \text{TFA in H}_2\text{O} \) = 45 / 55
Sample: Propionaldehyde

Mobile Phase: \( \text{CH}_3\text{CN} / 0.05\% \text{TFA in H}_2\text{O} \) = 70 / 30
Sample: Heptan-2-one
Labeling Reagent for Fluorescence Detection of Carbonyl Compounds

NBD-H (4-Hydrazino-7-nitro-2,1,3-benzoxadiazole Hydrazine) 100mg [A5557]

The compound A5557 is an HPLC fluorescence labeling reagent, which has a 2,1,3-benzoxadiazole skeleton and a hydrazino group, easily reacts with a carbonyl group to form the corresponding hydrazone. The labeling reagent itself is non-fluorescent, but the hydrazones after the reaction with carbonyl compounds have strong fluorescence. The resultant hydrazone is stable enough to reach the detector without any decomposition under reversed phase HPLC. An excellent chromatogram can be obtained by fluorescence detection at the excitation and emission wavelengths of 470 nm and 550 nm, respectively. Since their excitation and fluorescence wavelengths are at longer wavelengths, detection has less interference by contaminants, and a highly sensitive detection can be done because of its high reactivity.

Application example:
[Aldehydes or ketones] ¹)
250 μM of the labeling reagent A5557 and 1.7 μM propionaldehyde are added to acetonitrile containing 0.025% TFA, and reacted at room temperature for 1 h, use it as the HPLC sample. For example, the detection limit for propionaldehyde is 35 fmol.

Reference
Chromatogram of aldehydes and ketones as NBD-H derivatives

Column: Kaseisorb LC ODS Super
Column Size: 4.6mm I.D. × 150mm
Detection: Fluorescence λex 470 nm λem 550 nm
Temperature: 30 ºC
Flow Rate: 1mL / min

Mobile Phase: CH₃CN / 0.05% TFA in H₂O = 50 / 50
Sample: Propionaldehyde

Mobile Phase: CH₃CN / 0.05% TFA in H₂O = 75 / 25
Sample: Heptan-2-one
Labeling Reagent for Fluorescence Detection
of Alcohols, Amines and Thiols

DBD-COCI
[4-(N,N-Dimethylaminosulfonyl)-7-(N-chloroformylmethyl-N-methylamino)-2,1,3-benoxadiazole] 100mg [A5558]

The compound A5558 is an HPLC fluorescence labeling reagent, which reacts with many kinds of nucleophilic groups under mild conditions. The reaction examples are shown in the table below.

These resulting compounds are stable, and can reach the detector without any decomposition under reversed phase HPLC, thus excellent chromatograms can be obtained by fluorescence detection.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Examples</th>
<th>Reaction Conditions</th>
<th>Wavelengths (nm)</th>
<th>Detection Limits (fmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alcohols</td>
<td>Androsterone</td>
<td>60 ºC, 30 min</td>
<td>443, 546</td>
<td>38</td>
</tr>
<tr>
<td>α-Oxyacids</td>
<td>Mandelic acid</td>
<td>60 ºC, 15 min</td>
<td>442, 551</td>
<td>125</td>
</tr>
<tr>
<td>Phenols</td>
<td>Estrone</td>
<td>60 ºC, 15 min</td>
<td>440, 543</td>
<td>40</td>
</tr>
<tr>
<td>Amines</td>
<td>Benzylamine</td>
<td>r.t. or 60 ºC, 15 min</td>
<td>445, 555</td>
<td>89</td>
</tr>
<tr>
<td>Aromatic</td>
<td>Phenetidine</td>
<td>60 ºC, 15 min</td>
<td>443, 553</td>
<td>56</td>
</tr>
<tr>
<td>Thiols</td>
<td>2-Mercapto-N-(2-naphthyl)-acetamide</td>
<td>r.t.</td>
<td>437, 544</td>
<td>103</td>
</tr>
</tbody>
</table>

Application example:
10 μL of 25 mM labeling reagent A5558 in dry benzene is mixed with 10 μL of 0.5 mM androsterone in dry benzene (containing 0.5 mM quinuclidine*), and incubated at 60 ºC for 30 min. The reaction solution is quenched with 980 μL of 50% acetonitrile solution containing 1% acetic acid, use it as the HPLC sample solution.

*For primary alcohols, quinuclidine is not necessarily needed.
Chromatogram of alcohol and amine as DBD-COCl derivatives

Column : Kaseisorb LC ODS Super
Column Size : 4.6 mm I.D. × 150 mm
Mobile Phase : CH₃CN / H₂O = 50 / 50
Detection : Fluorescence λex 450 nm
            λem 560 nm
Temperature : 40 °C
Flow Rate : 1 mL / min

Sample : Androsterone
Sample : Benzylamine

References
Labeling Reagent for Fluorescence Detection
of Chiral Carboxylic Acids

{(S)-(+)-DBD-APy}
\[\stackrel{\text{[A5560]}}{\text{[=(S)-(+)-4-\text{(N,N-Dimethylaminosulfonyl)-7-\text{(3-aminopyrrolidin-1-yl)-}\text{2,1,3-benzoxadiazole}]}]}\]

The compound \textbf{A5560} is an optically active HPLC fluorescence labeling reagent, which has a 2,1,3-benzoxadiazole skeleton and an amino group, and is used for optical purity determination of carboxylic acids. Labeling of racemic carboxylic acids can be done by using a mild condition such as the Mukaiyama-Corey method, and produces diastereomers without inducing racemization. These diastereomers can be separated by reversed phase HPLC, and an excellent chromatogram can be obtained by fluorescence detection at the excitation and emission wavelengths of 470 nm and 580 nm, respectively. Since their excitation and fluorescence wavelengths are at longer wavelengths, detection has less interference by contaminants. A highly sensitive analysis can be done by peroxoxyxalate chemiluminescence detection.

\[\text{ex}=470 \text{ nm}\]
\[\text{em}=580 \text{ nm}\]

Application example:

1) Add 0.1 mL of 10 mM labeling reagent \textbf{A5560} / acetonitrile solution, 0.25 mL of 2 \textmu M carboxylic acid / acetonitrile solution, and 0.15 mL of 10 mM 2,2'-dipyridyl disulfide-triphenylphosphine / acetonitrile solution to a vessel, and react the mixture at room temperature for 4 h. Use the resultant as an HPLC sample solution.

For example, the detection limit (S/N=2) for naproxen is 10 fmol.

References
Chromatogram of carboxylic acid enantiomers as (S)-(+-)DBD-APy derivatives

Column : Kaseisorb LC ODS Super
Column Size : 4.6 mm I.D. × 150 mm
Detection : Fluorescence λex 470 nm
             λem 580 nm
Temperature : 40 °C
Flow Rate : 1 mL / min

Mobile Phase :
CH$_3$CN / H$_2$O = 50 / 50
Sample :
1. (S)-(+-)-Naproxen

Mobile Phase :
CH$_3$CN / H$_2$O = 60 / 40
Sample :
1. (S)-(+-)-Ibuprofen
2. (R)-(+-)-Ibuprofen
Labeling Reagent for Fluorescence Detection of Chiral Carboxylic Acids

(R)-(-)-DBD-APy
[=(R)-(-)-4-(N,N-Dimethylaminosulfonyl)-7-(3-aminopyrrolidin-1-yl)-2,1,3-benzoxadiazole]

The compound A5561 is an optically active HPLC fluorescence labeling reagent, which has a 2,1,3-benzoxadiazole skeleton and an amino group, and is used for optical purity determination of carboxylic acids. Labeling of racemic carboxylic acids can be done by using a mild condition such as the Mukaiyama-Corey method, and produces diastereomers without inducing racemization. These diastereomers can be separated by reversed phase HPLC, and an excellent chromatogram can be obtained by fluorescence detection at the excitation and emission wavelengths of 470 nm and 580 nm, respectively. Since their excitation and fluorescence wavelengths are at longer wavelengths, detection has less interference by contaminants. A highly sensitive analysis can be done by peroxyoxalate chemiluminescence detection.

Application example:
Add 0.1 mL of 10 mM labeling reagent A5561 / acetonitrile solution, 0.25 mL of 2 μM carboxylic acid / acetonitrile solution, and 0.15 mL of 10 mM 2,2'-dipyridyl disulfide-triphenylphosphine / acetonitrile solution to a vessel, and react the mixture at room temperature for 4 h. Use the resultant as an HPLC sample solution.
For example, the detection limit (S/N=2) for naproxen is 10 fmol.

References
Chromatogram of carboxylic acid enantiomers as \((R)-(-)-\text{DBD-APy derivatives}\)

- **Column**: Kaseisorb LC ODS Super
- **Column Size**: 4.6 mm I.D. \(\times\) 150 mm
- **Detection**: Fluorescence \(\lambda_{\text{ex}}\) 470 nm \(\lambda_{\text{em}}\) 580 nm
- **Temperature**: 40 \(^\circ\)C
- **Flow Rate**: 1 mL / min

**Mobile Phase**:

\[\text{CH}_3\text{CN} / \text{H}_2\text{O} = 50 / 50\]

**Sample**:
1. \((S)-(+)\)-Naproxen

**Mobile Phase**:

\[\text{CH}_3\text{CN} / \text{H}_2\text{O} = 60 / 40\]

**Sample**:
1. \((R)-(-)\)-Ibuprofen
2. \((S)-(+)\)-Ibuprofen
Labeling Reagent for Fluorescence Detection
for Chiral Carboxylic Acids

\[ \text{(S)-(+)\text{-NBD\text{-APy}}} \]

\[ \text{[= (S)-(+) -4-Nitro-7-(3-aminopyrrolidin-1-yl)-2,1,3-benzoxadiazole]} \]

The compound \textbf{A5562} is an optically active HPLC fluorescence labeling reagent, which has a 2,1,3-benzoxadiazole skeleton and an amino group, and is used for optical purity determination of carboxylic acids. Labeling of racemic carboxylic acids can be done by using a mild condition such as the Mukaiyama-Corey method, and produces diastereomers without inducing racemization. These diastereomers can be separated by reversed phase HPLC, and an excellent chromatogram can be obtained by fluorescence detection at the excitation and emission wavelengths of 470 nm and 540 nm, respectively. Since their excitation and fluorescence wavelengths are at longer wavelengths, detection has less interference by contaminants. A highly sensitive detection can be done by using laser induced fluorescence detector.

\[ \text{Application example:} \]

Add 0.1 mL of 10 mM labeling reagent \textbf{A5562} / acetonitrile solution, 0.25 mL of 2 \mu M carboxylic acid / acetonitrile solution, and 0.15 mL of 10 mM 2,2'-dipyridyl disulfide-triphenylphosphine / acetonitrile solution to a vessel, and react the mixture at room temperature for 4 h. Use the resultant as an HPLC sample solution.

For example, the detection limit (S/N=2) for naproxen is 15 fmol.

\[ \text{References} \]

Chromatogram of carboxylic acid enantiomers as \((S)\)-\((+)-NBD-APy\) derivatives

Column : Kaseisorb LC ODS Super
Column Size : 4.6 mm I.D. × 150 mm
Mobile Phase : \(\text{CH}_3\text{CN} / \text{H}_2\text{O} = 50 / 50\)
Detection : Fluorescence \(\lambda_{\text{ex}} 470 \text{ nm}\)
\(\lambda_{\text{em}} 540 \text{ nm}\)
Temperature : 40 °C
Flow Rate : 1 mL / min

Sample : 1. \((S)\)-(+) Naproxen
2. \((R)\)-(-) Ibuprofen

Sample : 1. \((S)\)-(+) Ibuprofen
2. \((R)\)-(+) Ibuprofen
Labeling Reagent for Fluorescence Detection of Chiral Carboxylic Acids

\((R)-(\cdot)-NBD-APy\)  
\([-=(R)-(\cdot)-4-Nitro-7-(3-aminopyrrolidin-1-yl)-2,1,3-benzoxadiazole]\)

The compound \textbf{A5563} is an optically active HPLC fluorescence labeling reagent, which has a 2,1,3-benzoxadiazole skeleton and an amino group, and is used for optical purity determination of carboxylic acids. Labeling of racemic carboxylic acids can be done by using a mild condition such as the Mukaiyama-Corey method, and produces diastereomers without inducing racemization. These diastereomers can be separated by reversed phase HPLC, and an excellent chromatogram can be obtained by fluorescence detection at the excitation and emission wavelengths of 470 nm and 540 nm, respectively. Since their excitation and fluorescence wavelengths are at longer wavelengths, detection has less interference by contaminants. A highly sensitive detection can be done by using laser induced fluorescence detector.

\[\text{OHC} \quad \text{R} \quad \text{NH}_2 \quad \text{NO}_2 \quad \text{N} \quad \text{O} \quad \text{N} \quad \text{O} \]

\[\text{ex}=470 \text{ nm} \quad \text{em}=540 \text{ nm}\]

\[\text{CH}_3\text{CN, r.t.}, 2,2'-\text{Dipyridyldisulfide - Ph}_3\text{P}\]

Application example:

Add 0.1 mL of 10 mM labeling reagent 1 / acetonitrile solution, 0.25 mL of 2 \(\mu\)M carboxylic acid / acetonitrile solution, and 0.15 mL of 10 mM 2,2'-dipyridyl disulfide-triphenylphosphine / acetonitrile solution to a vessel, and react the mixture at room temperature for 4 h. Use the resultant as an HPLC sample solution.

For example, the detection limit (S/N=2) for naproxen is 15 fmol.

References

Chromatogram of carboxylic acid enantiomers as (R)-(−)-NBD-APy derivatives

Column: Kaseisorb LC ODS Super
Column Size: 4.6 mm I.D. × 150 mm
Mobile Phase: CH₃CN / H₂O = 50 / 50
Detection: Fluorescence λ<sub>ex</sub> 470 nm, λ<sub>em</sub> 540 nm
Temperature: 40 °C
Flow Rate: 1 mL / min

Sample: 1. (S)-(+)–Naproxen
2. (S)-(+)–Ibuprofen

Sample: 1. (R)-(−)–Ibuprofen
2. (S)-(+)–Ibuprofen
Labeling Reagent for Fluorescence Detection of Chiral Alcohols and Amines

(S)-(−)-DBD-Pro-COCl 100mg [A5564]

\[=(S)-(\cdot)-4-(N,N\text{-Dimethylaminosulfonyl})-7-(2\text{-chloroformylpyrrolidin-1-yl})-2,1,3\text{-benzoxadiazole}\]

The compound **A5564** is an HPLC fluorescence labeling reagent for optical purity determination, which easily reacts with optical active alcohols or amines to form the corresponding esters or amides, respectively. The resultant esters or amides are stable and can reach the detector without any decomposition under reversed and normal phase HPLC. An excellent chromatogram can be obtained by fluorescence detection at the excitation and emission wavelengths of 450 nm and 560 nm, respectively. And the diastereomers derived from racemic alcohol and **A5564** can be separated by HPLC (The separation factor \(\alpha\): 2-hexanol = 1.2). Since no racemization can occur with derivatization reaction, it is possible to change an elution order of the labeled diastereomers by selecting the enantiomer \([R]\)-(+)\-DBD-Pro-COCl\] of **A5564**. The detection limit for the alcohols is sub-picomol. A highly sensitive analysis can be done by peroxyoxalate chemiluminescence detection.

**Application example:**

[Secondary alcohols] ¹)

Add 1 mL of 10 mM labeling reagent **A5564** / dry benzene solution and 1 mL of 2 mM alcohol / dry benzene (containing 1% of pyridine) solution to a vessel. Close the cap of the reaction vessel and incubate the mixture at 80 °C for 3 h. After cooling to room temperature, excess of **A5564** is removed by liquid-liquid extraction (e.g. washing with 5% NaHCO₃ solution) or solid phase extraction. Use the resultant as an HPLC sample solution.

**References**

Chromatogram of alcohol enantiomers as (S)-(-)-DBD-Pro-COCl derivatives

Column: Kaseisorb LC 60-5
Column Size: 4.6 mmI.D. × 150 mm
Mobile Phase: CH₃CN / H₂O = 60 / 40
Temperature: 25 °C
Detection: Fluorescence λex 450 nm, λem 560 nm
Flow Rate: 1 mL / min
Sample: 1. (S)-(+).Hexan-2-ol
2. (R)-(-)-Hexan-2-ol
3. (S)-(+) Nonan-2-ol
4. (R)-(-)-Nonan-2-ol

Column: Kaseisorb LC ODS Super
Column Size: 4.6 mmI.D. × 150 mm
Mobile Phase: Hexane / AcOEt = 80 / 20
Temperature: 25 °C
Detection: Fluorescence λex 450 nm, λem 560 nm
Flow Rate: 1 mL / min
Sample: 1. (S)-(+) Hexan-2-ol
2. (R)-(-)-Hexan-2-ol
3. (S)-(+) Nonan-2-ol
4. (R)-(-)-Nonan-2-ol
5. Hexan-2-ol
Labeling Reagent for Fluorescence Detection
of Chiral Alcohols and Amines

\[(R)-(+)-\text{DBD-Pro-COCI} \quad 100\text{mg [A5565]}
\]

\implies [(R)-(+)-4-(N,N-Dimethylaminosulfonyl)-7-(2-chloroformylypyrrolidin-1-yl)-2,1,3-benzoxadiazole]

\[\text{A5565}\]

The compound \textbf{A5565} is an HPLC fluorescence labeling reagent for optical purity determination, which easily reacts with optical active alcohols or amines to form the corresponding esters or amides, respectively. The resultant esters or amides are stable and can reach the detector without any decomposition under reversed and normal phase HPLC. An excellent chromatogram can be obtained by fluorescence detection at the excitation and emission wavelength of 450 nm and 560 nm, respectively. And the diastereomers derived from racemic alcohol and \textbf{A5565} can be separated by HPLC (The separation factor \(\alpha\): 2-hexanol = 1.2). Since no racemization can occur with derivatization reaction, it is possible to change an elution order of the labeled diastereomers by selecting the enantiomer \(\textbf{(S)-(-)-DBD-Pro-COCI}\) of \textbf{A5565}. The detection limit for the alcohols is sub-picomol. A highly sensitive analysis can be done by peroxyoxalate chemiluminescence detection.

Application example:

\[\text{[Secondary alcohols]} \]

1) Add 1 mL of 10 mM labeling reagent \textbf{A5565} / dry benzene solution, 1 mL of 2 mM alcholol / dry benzene (containing 1% of pyridine) solution to a vessel. Close the cap of the reaction vessel and incubate the mixture at 80 °C for 3 h. After cooling to room temperature, excess of \textbf{A5565} is removed by liquid-liquid extraction (e.g. washing with 5% NaHCO\(_3\) solution) or solid phase extraction. Use the resultant as an HPLC sample solution.

References

Chromatogram of alcohol enantiomers as (R)-(+)−DBD-Pro-COCI derivatives

**Column**: Kaseisorb LC 60-5
**Column Size**: 4.6 mm I.D. × 150 mm
**Mobile Phase**: Hexane / AcOEt = 80 / 20
**Temperature**: 25 °C
**Detection**: Fluorescence $\lambda_{ex}$ 450 nm $\lambda_{em}$ 560 nm
**Flow Rate**: 1 mL / min

**Sample**:
1. (R)-(−)-Hexan-2-ol
2. (S)-(−)-Hexan-2-ol
3. (R)-(−)-Nonan-2-ol
4. (S)-(−)-Nonan-2-ol

**Column**: Kaseisorb LC ODS Super
**Column Size**: 4.6 mm I.D. × 150 mm
**Mobile Phase**: CH$_3$CN / H$_2$O = 60 / 40
**Temperature**: 25 °C
**Detection**: Fluorescence $\lambda_{ex}$ 450 nm $\lambda_{em}$ 560 nm
**Flow Rate**: 1 mL / min
**Sample**:
5. Hexan-2-ol
Labeling Reagent for Fluorescence Detection  
of Chiral Alcohols and Amines

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

\[
\text{[=}(R)-(+)-4\text{-Nitro-7-(2-chloroformylpyrrolidin-1-yl)-2,1,3-benzoxadiazole}]
\]

The compound A5566 is an HPLC fluorescence labeling reagent for optical purity determination, which easily reacts with optical active alcohols or amines to form the corresponding esters or amides, respectively. The resultant esters or amides are stable and can reach the detector without any decomposition under reversed and normal phase HPLC. An excellent chromatogram can be obtained by fluorescence detection at the excitation and emission wavelengths of 470 nm and 540 nm, respectively. And the diastereomers derived from racemic alcohol or amine and A5566 can be separated by HPLC (The separation factor \( \alpha \): 2-hexanol and 1-phenylethylamine = 1.2 and 1.37, respectively). Since no racemization can occur with derivatization reaction, it is possible to change an elution order of the labeled diastereomeres by selecting the enantiomer [(S)-(-)-NBD-Pro-COCl] of A5566. The detection limit for the alcohols is sub-picomol. A highly sensitive detection can be done by using laser induced fluorescence detector.

Application example:

Add 0.5 mL of 40 mM labeling reagent A5566 / dry benzene solution and 0.5 mL of 1 mM alcholol (or amine) / dry benzene (containing 2% of pyridine) solution to a vessel. Close the cap of the reaction vessel and incubate the mixture at 80 °C for 1~2 h (50 °C for 1h, in the case of amine). After cooling to room temperature, excess of A5566 is removed by liquid-liquid extraction (e.g. washing with 5% NaHCO₃ solution) or solid phase extraction. Use the resultant as an HPLC sample solution.

Reference

Chromatogram of alcohol and amine enantiomers as (R)-(±)-NBD-Pro-COCl derivatives

<table>
<thead>
<tr>
<th>Column</th>
<th>Kaseisorb LC 60-5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Column Size</td>
<td>4.6 mm I.D. × 150 mm</td>
</tr>
<tr>
<td>Mobile Phase</td>
<td>Hexane / AcOEt = 80 / 20</td>
</tr>
<tr>
<td>Temperature</td>
<td>40 °C</td>
</tr>
<tr>
<td>Detection</td>
<td>Fluorescence λ&lt;sub&gt;ex&lt;/sub&gt; 470 nm, λ&lt;sub&gt;em&lt;/sub&gt; 540 nm</td>
</tr>
<tr>
<td>Flow Rate</td>
<td>1 mL/min</td>
</tr>
<tr>
<td>Sample</td>
<td>1. (R)-(−)-Hexan-2-ol</td>
</tr>
<tr>
<td></td>
<td>2. (S)-(−)-Hexan-2-ol</td>
</tr>
<tr>
<td></td>
<td>3. (R)-(−)-Heptan-2-ol</td>
</tr>
<tr>
<td></td>
<td>4. (S)-(−)-Heptan-2-ol</td>
</tr>
<tr>
<td></td>
<td>5. (R)-(+)Phenylethylamine</td>
</tr>
<tr>
<td></td>
<td>6. (S)-(−)-Phenylethylamine</td>
</tr>
</tbody>
</table>

Mobile Phase : Hexane / AcOEt = 55 / 45
Sample : 5. (R)-(+)Phenylethylamine
6. (S)-(−)-Phenylethylamine
Labeling Reagent for Fluorescence Detection
of Chiral Alcohols and Amines

(S)-(−)-NBD-Pro-COCI
[=(S)-(−)-4-Nitro-7-(2-chloroformylypyrrolidin-1-yl)-2,1,3-benzoxadiazole]

![Chemical Structure](image.png)

The compound **A5567** is an HPLC fluorescence labeling reagent for optical purity determination, which easily reacts with optical active alcohols or amines to form the corresponding esters or amides, respectively. The resultant esters or amides are stable and can reach the detector without any decomposition under reversed and normal phase HPLC. An excellent chromatogram can be obtained by fluorescence detection at the excitation and emission wavelengths of 470 nm and 540 nm, respectively. And the diastereomers derived from racemic alcohol or amine and **A5567** can be separated by HPLC (The separation factor α: 2-hexanol and 1-phenylethylamine = 1.2 and 1.37, respectively). Since no racemization can occur with derivatization reaction, it is possible to change an elution order of the labeled diastereomers by selecting the enantiomer [(R)-(+)−NBD-Pro-COCI] of **A5567**. The detection limit for the alcohols is sub-picomol. A highly sensitive detection can be done by laser induced fluorescence detector.

**Application example:**

Add 0.5 mL of 40 mM labeling reagent **A5567** / dry benzene solution, 0.5 mL of 1 mM alcohol (or amine) / dry benzene (containing 2% of pyridine) solution to a vessel. Close the cap of the reaction vessel and incubate the mixture at 80 ºC for 1~2 h (50 ºC for 1 h, in the case of amine). After cooling to room temperature, excess of **A5567** is removed by liquid-liquid extraction (e.g. washing with 5% NaHCO₃ solution) or solid phase extraction. Use the resultant as an HPLC sample solution.

**Reference**

Chromatogram of alcohol and amine enantiomers
as (S)-(−)-NBD-Pro-COCI derivatives

Column : Kaseisorb LC 60-5
Column Size : 4.6 mm I.D. × 150 mm
Mobile Phase : Hexane / AcOEt = 80 / 20
Temperature : 40 °C
Detection : Fluorescence \( \lambda_{ex} 470 \, \text{nm} \)
\( \lambda_{em} 540 \, \text{nm} \)
Flow Rate : 1 mL / min
Sample : 1. \((S)\)-(+)‐Hexan‐2‐ol
2. \((R)\)-(−)‐Hexan‐2‐ol
3. \((S)\)-(+)‐Heptan‐2‐ol
4. \((R)\)-(−)‐Heptan‐2‐ol

Mobile Phase : Hexane / AcOEt = 55 / 45
Sample : 5. \((S)\)-(−)‐Phenylethylamine
6. \((R)\)-(+)‐Phenylethylamine
Labeling Reagent for Fluorescence Detection
of Chiral Amines and Thiols

\((R)-(\cdot)\)-DBD-Py-NCS
\([=\{(R)-(\cdot)-4-(N,N-Dimethylaminosulfonyl)-7-(3-isothiocyanatopyrrolidin-1-yl)-2,1,3-benzoxadiazole\}\]

The compound \textbf{A5568} is an HPLC fluorescence labeling reagent for optical purity determination. This compound easily reacts with amino or mercapto groups, which are directly linked to the asymmetric carbon atom and produces diastereomers of thiourea or dithiocarbamate. These diastereomers can be separated by reversed phase HPLC, and an excellent chromatogram can be obtained by fluorescence detection at the excitation and emission wavelengths of 460 nm and 550 nm, respectively. [The detection limit: thiopronine = 0.5 pmol (S/N = 2)]

Since both \((R)\)- and \((S)\)-isomers of the derivatization reagents are commercially available on the market, it is possible to change an elution order of the derivatized diastereomers by selecting either enantiomer of the derivatization reagent. Thus, an enantiomer of the amino-compound, whose existing quantity is very small, can be eluted out first and quantified with a high precision. Moreover, there are reports for the application of these isomers to Edman Degradation.

Application example:

Add 10 μL of 5 mM HPLC labeling reagent \textbf{A5568} / acetonitrile solution in 10 μL of 1 mM amine / acetonitrile-H\textsubscript{2}O (1:1) solution (containing 2% triethylamine) to a vessel, close the cap of the reaction vessel and incubate the mixture at 55 °C for 10 min. Then, add 480 μL of a mixture solution of 1 M acetic acid and acetonitrile-H\textsubscript{2}O (1:1) solution, and dilute this reactant mixture 10x by acetonitrile. Use 5 μL of this diluted solution as an HPLC sample solution.
Chromatogram of amines as \((R)\)-(\-\-\-)DBD-Py-NCS derivatives

Column : Kaseisorb LC ODS Super
Column Size : 4.6 mm I.D. x 150 mm
Mobile Phase : \(\text{CH}_3\text{CN} / H_2O = 40 / 60\)
containing 0.05\% TFA
Detection : Fluorescence \(\lambda_{\text{ex}}\) 460 nm
\(\lambda_{\text{em}}\) 550 nm
Temperature : Ambient
Flow Rate : 1 mL / min
Sample : 1. \((R)\)-1-Phenylethylamine
2. \((S)\)-1-Phenylethylamine

References
Labeling Reagent for Fluorescence Detection of Chiral Amines and Thiols

\((S)-(+)\)-DBD-Py-NCS
\([=S(+)\text{-}4-(N,N\text{-}Dimethylaminosulfonyl)\text{-}7\text{-}(3\text{-}isothiocyanatopyrrolidin-1-yl)\text{-}2,1,3\text{-}benzoxadiazole}\]

The compound \textbf{A5569} is an HPLC fluorescence labeling reagent for optical purity determination. This compound easily reacts with amino or mercapto groups, which are directly linked to the asymmetric carbon atom and produces diastereomers of thiourea or dithiocarbamate. These diastereomers can be separated by reversed phase HPLC, and an excellent chromatogram can be obtained by fluorescence detection at the excitation and emission wavelengths of 460 nm and 550 nm, respectively. [The detection limit: thiopronine = 0.5 pmol (S/N = 2)]

Since both \((R)\)- and \((S)\)-isomers of the derivatization reagents are commercially available on the market, it is possible to change an elution order of the derivatized diastereomers by selecting either enantiomer of the derivatization reagent. Thus, an enantiomer of the amino-compound, whose existing quantity is very small, can be eluted out first and quantified with a high precision. Moreover, there are reports for the application of these isomers to Edman Degradation.

**Application example:**

Add 10 \(\mu\)L of 5 mM HPLC labeling reagent \textbf{A5569} / acetonitrile solution in 10 \(\mu\)L of 1 mM amine / acetonitrile-H\(_2\)O (1:1) solution (containing 2% triethylamine) to a vessel, close the cap of the reaction vessel and incubate the mixture at 55 \(^\circ\)C for 10 min. Then, add 480 \(\mu\)L of a mixture solution of 1 M acetic acid and acetonitrile-H\(_2\)O (1:1) solution, and dilute this reactant mixture 10x by acetonitrile. Use 5 \(\mu\)L of this diluted solution as an HPLC sample solution.
Chromatogram of amines as (S)-(+)~DBD-Py-NCS derivatives

- Column : Kaseisorb LC ODS Super
- Column Size : 4.6 mm i.d. × 150 mm
- Mobile Phase : CH₃CN / H₂O = 40 / 60
  containing 0.05% TFA
- Detection : Fluorescence λex 460 nm
  λem 550 nm
- Temperature : Ambient
- Flow Rate : 1.0 mL / min
- Sample : 1. (S)-1-Phenylethylamine
  2. (R)-1-Phenylethylamine

References
Labeling Reagent for Fluorescence Detection
of Carboxylic Acids

4-Bromomethyl-6,7-dimethoxycoumarin

The compound A5570 is an HPLC fluorescence labeling reagent, which has a bromomethyl group, and easily reacts with a carboxyl group to form the corresponding ester in the presence of a base. The resultant ester is stable enough to reach the detector without any decomposition under reversed phase HPLC. Furthermore, it has a characteristic fluorescence based on a coumarin skeleton, thus an excellent chromatogram can be obtained by fluorescence detection at the excitation and emission wavelengths of 340 nm and 425 nm, respectively.

Application examples:

[Fatty acids]
1) Dissolve 0.01 g of the sample in 0.1 mL of acetone. The solution is neutralized by the addition of 10% KOH / methanol. To the resultant solution, add an acetone solution with an excess amount of labeling reagent A5570, 18-crown 6-ether, and potassium carbonate. Close the cap of the reaction vessel and incubate the mixture at 70 ºC for 30 min. Cool to room temperature and use it as an HPLC sample solution.

[Others]
Prostaglandins¹, bile acids¹, proteins², nucleic acids³

References
Chromatogram of fatty acids
as 6,7-Dimethoxycoumarin 4-methyl esters

Column : Kaseisorb LC ODS Super
Column Size : 4.6 mmID. × 150 mm
Mobile Phase : CH₃CN
Detection : Fluorescence λex 340 nm
           λem 425 nm
Temperature : Ambient
Flow Rate : 1 mL / min
Sample : 1. Linolic Acid
         2. Oleic Acid
Labeling Reagent for Fluorescence Detection of Carboxylic Acids

NBD-CO-Hz

100mg [A5573]

[=4-(N-Hydrazinocarbonylmethyl-N-methylamino)-7-nitro-2,1,3-benzoxadiazole]

The compound A5573, an HPLC fluorescence labeling reagent, which has a 2,1,3-benzoxadiazole skeleton and a hydrazino group, easily reacts with a carboxyl group to form the corresponding carbohydrazide in the presence of a condensing agent. The resultant carbohydrazide is stable for at least one week at 4 ºC. The carbohydrazide derivatives can be analyzed by reversed phase HPLC, and an excellent chromatogram can be obtained by fluorescence detection at the excitation and emission wavelengths of 475 nm and 530 nm, respectively. [The detection limit = 2~4 fmol (S/N = 3)]

Application example:
Add 50 μL of carboxylic acid / DMF solution, 50 μL of 1.0 M EDC aqueous solution, 50 μL of 20% pyridine aqueous solution and 20 mM HPLC labeling reagent A5573 / DMF solution to a vessel, and incubate the mixture at room temperature for 2 h. Dilute this reactant mixture 10x with the mobile phase solution, and use 1 μL of this diluted solution as an HPLC sample solution.

Reference
Chromatogram of non-steroidal anti-inflammatory drugs as NBD-CO-Hz derivatives

Column: Kaseisorb LC ODS 2000
Column Size: 4.6 mm i.d. × 150 mm
Mobile Phase: CH₃OH / H₂O = 70 / 30
Detection: Fluorescence λex 475 nm, λem 530 nm
Temperature: Ambient
Flow Rate: 1.0 mL / min
Sample: 1. Ketoprofen
2. Flurbiprofen
3. Ibuprofen
Labeling Reagent for Fluorescence Detection of Carboxylic Acids

**DBD-ED**

[=4-(N,N-Dimethylaminosulfonyl)-7-(2-aminoethylamino)-2,1,3-benzoxadiazole] 100mg [A5574]

The compound A5574, an HPLC fluorescence labeling reagent, which has a 2,1,3-benzoxadiazole skeleton and an amino group, easily reacts with a carboxyl group to form the corresponding amide in the presence of a condensing agent. The resultant amide is stable enough to reach the detector without any decomposition under reversed phase HPLC. An excellent chromatogram can be obtained by fluorescence detection at the excitation and emission wavelengths of 450 nm and 560 nm, respectively. Since their excitation and fluorescence wavelengths are at longer wavelengths, detection has less interference from contaminants. Short-chain fatty acids are detectable and determinable reproducibly with a detection limit on the order of fmol. A highly sensitive detection can be done by using chemiluminescence.

**Application example:**

Add 50 μL of mixed fatty acid / diethyl ether solution, 50 μL of 50 mM labeling reagent A5574 / acetonitrile solution, 50 μL of triphenylphosphine / acetonitrile solution and 50 μL of 2,2'-dipyridyl disulfide / acetonitrile solution to a vessel. This mixture is kept in the dark at room temperature. Dilute this reactant mixture 100x by acetonitrile, and use 10 μL of this diluted solution as an HPLC sample solution.

**References**

Chromatogram of fatty acids as DBD-ED derivatives

Column: Kaseisorb LC ODS 2000
Column Size: 4.6 mm i.D. × 150 mm
Mobile Phase: CH₃CN / H₂O = 95 / 5
Detection: Fluorescence λex 450 nm, λem 560 nm
Temperature: 40 °C
Flow Rate: 1.0 mL / min
Sample:
1. Linolenic Acid
2. Linolic Acid
3. Oleic Acid
4. Stearic Acid
Labeling Reagent for Fluorescence Detection
for Amines

DBD-NCS
[=4-(N,N-Dimethylamino sulfonyl)-7-isothiocyanato-2,1,3-benzoxadiazole]

The compound **A5575** is an HPLC fluorescence labeling reagent, which has a 2,1,3-benzoxadiazole skeleton and an isothiocyanate group, and easily reacts with an amino group to form the corresponding thiourea. The resultant thiourea is stable enough to reach the detector without any decomposition under reversed phase HPLC. An excellent chromatogram can be obtained by fluorescence detection at the excitation and emission wavelengths of 384 nm and 520 nm, respectively. The detection limit for its quantity is an order of sub-picomol (S/N = 3). **A5575** itself does not fluoresce but shows an excellent stability in forms of both crystal and solution, and its derivatives are also stable. This compound can be used for amino acid sequence analysis (Edman Degradation) by binding with the N-terminal amino acid of peptides or proteins, followed by acid treatment.

\[ \text{DBD-CA} \]

**AZ-576**
Application example:
[Method by Manual Edman Degradation]
Peptide (insulin Chain B 500 pmol)

- Dissolve in 20 μL of 50% pyridine / H₂O.
- Add 5 μL of 1% triethylamine / CH₃CN and 10 μL of 20 mM HPLC labeling reagent A5575 / pyridine, and react the mixture at 50 ºC for 15 min under the atmosphere of inert gas.
- After cooling to room temperature, wash the reactant solution 3 times with 200 μL of heptane / dichlorormethane (6/4).
- Dry the washed solution at 50 ºC for 15 min by using a centrifugation evaporator.
- Add 30 μL of 1% BF₃•Et₂O / CH₃CN to the mixture and incubate the mixture at 50 ºC for 5 min.
- Further dry the reactant solution under nitrogen gas.
- Add 20 μL of H₂O, and then extract 2 times with 100 μL of benzene / AcOEt (1/4). (Aqueous phase) (Organic phase)
A peptide will be eluted out.

- Dry the extracted organic phase under nitrogen gas.
- Dissolve the mixture in 2 μL of CH₃CN.
- Add 8 μL of 0.4 M HCl and hydrolyze the mixture at 50 ºC for 5 min.
- Treat the reactant with 5 μL of 4 M HCl and 0.5 M NaNO₂ at room temperature for 10 min and oxidize it.
- Neutralize the reactant with 23 μL of 1 M NaNO₂, and remove an excessive oxidant by adding 20 μL of 0.15 M methionine.

Use 20 μL of this solution as an HPLC sample solution.

References
Labeling Reagent for Fluorescence Detection of Carboxylic Acids

AABD-SH (=4-Acetamido-7-mercapto-2,1,3-benzoxadiazole) 100mg [A5576]

The compound A5576, an HPLC fluorescence labeling reagent, which has a 2,1,3-benzoxadiazole skeleton and a mercapto group, easily reacts with a carboxyl group to form the corresponding thioester. A5576 itself fluoresces very little, but the thioester derivatives fluoresce highly. The resultant thioester is stable enough to reach the detector without any decomposition under reversed phase HPLC. An excellent chromatogram can be obtained by fluorescence detection at the excitation and emission wavelengths of 368 nm and 524 nm, respectively. [The detection limit = 10~20 fmol (S/N = 3)]

Application example:
Add 20 μL of mixed fatty acid / acetonitrile solution, 20 μL of 20 mM labeling reagent A5576 / dichloromethane solution, 20 μL of triphenylphosphine / acetonitrile solution and 20 μL of 2,2'-dipyridyl disulfide / acetonitrile solution to a 500 μL vessel, and the mixture is left at room temperature for 15 min. Dilute this reactant mixture with 20 μL of acetonitrile, and use 1 μL of this diluted solution as an HPLC sample solution.

Reference
Chromatogram of fatty acids as AABD-thio esters

- Chromatogram details:
  - Column: Kaseisorb LC ODS 2000
  - Column Size: 4.6 mm I.D. × 150 mm
  - Mobile Phase: CH$_3$OH
  - Detection: Fluorescence $\lambda_{ex}$ 368 nm, $\lambda_{em}$ 524 nm
  - Temperature: Ambient
  - Flow Rate: 1 mL / min
  - Sample: 1. Linolenic Acid, 2. Linoleic Acid, 3. Oleic Acid, 4. Stearic Acid

- Time scale: 0 - 10 minutes
Labeling Reagent for Fluorescence Detection
of Chiral Amines

(R)-(-)-NBD-Py-NCS
[= (R)-(-)-4-(3-Isothiocyanatopyrrolidin-1-yl)-7-nitro-2,1,3-benzoxadiazole]

The compound A5577 is an HPLC fluorescence labeling reagent for optical purity determination. This compound easily reacts with amino groups, which are directly linked to an asymmetric carbon atom, and produces diastereomers of thiourea. These diastereomers can be separated by reversed phase HPLC, and an excellent chromatogram can be obtained by fluorescence detection at the excitation and emission wavelengths of 488 nm and 590 nm, respectively.

Since both (R)- and (S)-isomers of the derivatization reagents are commercially available on the market, it is possible to change the elution order of the derivatized diastereomers by selecting either enantiomer of the derivatization reagent. Thus, an enantiomer of the amino-compound, whose existing quantity is very small, can be eluted out first and quantified with high precision. Moreover, there are reports for the application of these isomers to Edman Degradation.

Application example:
Add 10 μL of 5 mM HPLC labeling reagent A5577 / acetonitrile solution in 10 μL of 1 mM amine / acetonitrile-H₂O (1:1) solution (containing 2% triethylamine) to a vessel. Close the cap of the reaction vessel and incubate the mixture at 55 °C for 10 min. Then, add 480 μL of a mixture solution of 1 M acetic acid and acetonitrile-H₂O (1:1) solution, and dilute this reactant mixture 10x by acetonitrile. Use 5 μL of this diluted solution as an HPLC sample solution.
Chromatogram of amino acids as (R)-(−)-NBD-Py-NCS derivatives

Column: Kaseisorb LC ODS 2000
Column Size: 4.6 mm I.D. × 150 mm
Mobile Phase: CH$_3$CN / H$_2$O = 40 / 60 containing 0.05% TFA
Temperature: 30 ºC
Detection: Fluorescence $\lambda_{ex}$ 488 nm $\lambda_{em}$ 590 nm
Flow Rate: 1.0 mL / min

Sample:
1. D-Valine
2. L-Valine
3. D-Phenylalanine
4. L-Phenylalanine

References
Labeling Reagent for Fluorescence Detection
of Chiral Amines

\[(S)\text{-}(\pm)-\text{NBD-Py-NCS}\]
\[=\text{[}\text{(S)}\text{-}(\pm)-4\text{-}(\text{3-Isothiocyanatopyrrolidin-1-yl)}\text{-}7\text{-}\text{nitro-2,1,3-benzoxadiazole}]\]

The compound \textbf{A5578} is an HPLC fluorescence labeling reagent for optical purity determination. This compound easily reacts with amino groups, which are directly linked to an asymmetric carbon atom, and produces diastereomers of thiourea. These diasteromers can be separated by reversed phase HPLC, and an excellent chromatogram can be obtained by fluorescence detection at the excitation and emission wavelengths of 488 nm and 590 nm, respectively.

Since both \((R)\)- and \((S)\)-isomers of the derivatization reagents are commercially available on the market, it is possible to change the elution order of the derivatized diastereomers by selecting either enantiomer of the derivatization reagent. Thus, an enantiomer of the amino-compound, whose existing quantity is very small, can be eluted out first and quantified with high precision. Moreover, there are reports for the application of these isomers to Edman Degradation.

**Application example:**

Add 10 \(\mu\)L of 5 mM HPLC labeling reagent \textbf{A5578} / acetonitrile solution in 10 \(\mu\)L solution of 1 mM amine / acetonitrile-\(\text{H}_2\text{O}\) (1:1) solution (containing 2\% triethylamine) to a vessel. Close the cap of the reaction vessel and incubate the mixture at 55 \(^\circ\)C for 10 min. Then, add 480 \(\mu\)L of a mixture solution of 1 M acetic acid and acetonitrile-\(\text{H}_2\text{O}\) (1:1) solution, and dilute this reactant mixture 10x by acetonitrile. Use 5 \(\mu\)L of this diluted solution as an HPLC sample solution.
Chromatogram of amino acids as (S)-(+)—NBD-Py-NCS derivatives

Column : Kaseisorb LC ODS 2000  
Column Size : 4.6 mmI.D. × 150 mm  
Mobile Phase : CH₃CN / H₂O = 40 / 60  
containing 0.05% TFA  
Temperature : 30 °C  
Detection : Fluorescence λex 488 nm  
λem 590 nm  
Flow Rate : 1.0 mL / min

Sample : 1. L-Valine  
2. D-Valine  
3. L-Phenylalanine  
4. D-Phenylalanine

References
Labeling Reagent for Fluorescence Detection
of Amines and Alcohols

4-(4,5-Diphenyl-1H-imidazol-2-yl)benzoyl Chloride Hydrochloride (=DIB・HCl) 100mg [A5579]

The compound A5579 is an HPLC fluorescence labeling reagent, which easily reacts with amino groups and hydroxyl groups to form the corresponding amides and esters, respectively. These derivatives are stable for at least 24 h at room temperature, and can reach the detector without any decomposition under reversed phase HPLC. Each derivative can be separated with ODS columns, and the detection limits (S/N = 3) are from 0.6 to 5.2 fmol / 5 μL injection\(^1\). A5579 is used for the quantitative analysis of methamphetamine and the derivatives in hair\(^3\), which is known to preserve drugs for a long term, as well as in urine\(^1,2\).

Application example:

[Quantitative analysis for methamphetamine analogs]\(^2\)
10 μL of urine collected from a methamphetamine addict and 10 μL of acetic acid are put into an amber-glass vial and dried under a flow of nitrogen. 10 μL of carbonate buffer solution and 190 μL of 100 μM labeling reagent A5579 / acetone solution are added to the residue, reacted at room temperature for 10 min. Use it as an HPLC sample solution.

References
Chromatogram of amines as DIB derivatives

Column: Daisopak SP-120-5-ODS-BP
Column Size: 4.6 mm I.D. × 250 mm
Mobile Phase: CH$_3$CN / H$_2$O = 65 / 35
Detection: Fluorescence $\lambda_{ex}$ 330 nm $\lambda_{em}$ 440 nm
Flow Rate: 1.0 mL / min
Sample:
1. Phentermine
2. Fenfluramine

Time, min

0 5 10 15 20 25 30 35 40

1 2
Labeling Reagent for Fluorescence Detection of Carbonyl Compounds

1,3-Cyclohexanedione 5g [A5581]

The compound A5581 is an HPLC fluorescence labeling reagent, and can easily react with a carbonyl groups to form the corresponding decahydroacridine-1,8-dion (DHA) derivative. The resultant derivative is stable enough to reach the detector without any decomposition under reversed phase HPLC. An excellent chromatogram can be obtained by fluorescence detection analysis at the excitation and emission wavelengths of 366 nm and 440 nm, respectively.

Application example:
[Aliphatic aldehydes] 1,2)
5 mL of acetic acid and 10 g of ammonium acetate are dissolved in distilled water. Then 0.25 g of labeling reagent A5581 is added to the solution and shaken to prepare the derivatization reagent solution. 2 mL of this solution is added to 1 mL of aqueous solution (ethanol solution, in the case of long-chain aldehydes) containing 10~30 ng of an aliphatic aldehyde, and incubate at 60 ºC for 30 min. After cooling, use 1 μL of this solution as an HPLC sample.

References
Chromatogram of aldehydes as DHA derivatives

Column : Kaseisorb LC ODS-100-5
Column Size : 4.6 mm I.D. × 150 mm
Mobile Phase : CH₃OH / H₂O =
                40 / 60 → 90 / 10
                20 min. linear gradient
Detection : Fluorescence λex 366 nm
            λem 440 nm
Flow Rate : 1 mL / min
Sample : 1. Formaldehyde
         2. Acetaldehyde
         3. Propionaldehyde
         4. Butyraldehyde
         5. Valeraldehyde
         6. Hexylaldehyde
         7. Heptylaldehyde
Labeling Reagent for Fluorescence Detection
for Thiols

NAM [=N-(9-Acridinyl)maleimide]

The compound A5591 is an HPLC fluorescence labeling reagent, and can easily react with a mercapto group at room temperature. The resultant sulfide is stable enough to reach the detector without any decomposition under reversed phase HPLC. An excellent chromatogram can be obtained by fluorescence detection analysis at the excitation and emission wavelengths of 355 nm and 465 nm, respectively.

Application example:

[Thiols] 1-5
0.4 mL of 30% NaOH solution and 1 mL of 0.2 M boric acid buffer solution (pH 8.8) are added to 2 mL of 1 mM sample solution in water. To this solution, 0.5 mL of 10 mM labeling reagent A5591 / acetone solution is added and shaken, and reacted at room temperature for 30 min to use it as a HPLC sample.

References
Chromatogram of thiols as NAM derivatives

Column: Kaseisorb LC ODS-300-5
Column Size: 4.6 mm I.D. x 150 mm
Mobile Phase: 0.05 M Na₂HPO₄ / CH₃CN = 89 / 11 (pH 7.5)
Detection: Fluorescence λₑₓ 355 nm, λₑₘ 465 nm
Flow Rate: 1 mL / min
Sample: 1. N-Acetyl-L-cysteine  
          2. 2-Mercaptoethanol
Labeling Reagent for Fluorescence Detection
for Amines and Thiols

NBD-Cl (= 4-Chloro-7-nitro-2,1,3-benzoxadiazole) 1g 5g [A5592]

![Chemical Structure of NBD-Cl]

The compound A5592 which is an HPLC fluorescence labeling reagent having a 2,1,3-benzoxadiazole skeleton, can easily react with a secondary amine and thiol. The resultant derivative is stable enough to reach the detector without any decomposition under general reversed phase HPLC. An excellent chromatogram can be obtained by fluorescence detection analysis at the excitation and emission wavelengths of 460 nm and 535 nm, respectively.

**Application examples:**

**[Alkylamines]**

To 25~500 μL of a methanol solution containing an amine (1~20 μg), 4~8 eq. excess amount of 0.05% labeling reagent A5592 / methanol solution is added. After adding 50~100 μL of 0.1 M NaHCO₃, incubate at 55 ºC for 1~5 h. After cooling the reaction mixture to room temperature, use it as an HPLC sample.

**[Others]**

TLC and HPLC of N-methylcarbamates, N,N-dimethylcarbamates in agrichemicals²,³
Hydrolyze the carbamates to label the amine derivatives.
TLC of amphetamines in urine⁴,⁵, HPLC of prolines (precolumn derivatization method)⁶

**References**

Chromatogram of alkylamines as NBD derivatives

Column : Kaseisorb LC ODS-300-5
Column Size : 4.6 mm I.D. × 150 mm
Mobile Phase : CH₃CN / H₂O = 45 / 55
Detection : Fluorescence λex 460 nm  λem 535 nm
Flow Rate : 1 mL / min
Sample : 1. Propylamine
2. Butylamine
3. Amylamine
Labeling Reagent for Fluorescence Detection
of Amines and Thiols

NBD-F (=4-Fluoro-7-nitro-2,1,3-benzoxadiazole) 100mg [A5593]

The compound A5593 which is an HPLC fluorescence labeling reagent having a 2,1,3-benzoxadiazole skeleton, can easily react with amino or mercapto groups to form the corresponding derivatives. A5593 itself does not fluoresce, and its ethanol solution is relatively stable for a week in a refrigerator. The derivatives can reach the detector without any decomposition under reversed phase HPLC. An excellent chromatogram can be obtained by fluorescence detection at the excitation and emission wavelengths of 470 nm and 530 nm, respectively. Since their excitation and fluorescence wavelengths are at longer wavelengths, detection has less interference by contaminants. Thus, further highly sensitive detection can be done by using laser induced fluorescence detector. When the reagent is hydrolyzed (NBD-OH), its fluorescence can be erased under an acidic condition. Therefore, this hydrolyzed reagent can be used as a post column reaction reagent.

Application example:
[Amino acids] 2,3
To 10 μL of 50 μM amino acid standard solution, add 10 μL of 0.1 M boric acid buffer solution (pH 8.0) and 20 μL of 50 mM labeling reagent A5593 in ethanol solution, and incubate the mixture at 60 ºC for 1 min. Immediately cool it with ice bath, and add 460 μL of 5 mM HCl to the reactant solution. Use 10 μL of the solution as an HPLC sample.

References
Chromatogram of amino acids as NBD derivatives

Column: Kaseisorb LC ODS Super
Column Size: 4.6 mm I.D. x 250 mm
Temperature: 40 ºC
Detection: Fluorescence λex 470 nm λem 530 nm
Flow Rate: 1 mL / min

Mobile Phase:
CH$_3$OH / THF / 0.1 M Phosphate buffer (pH 6.0)
= 20 / 20 / 60

Mobile Phase:
CH$_3$OH / THF / 0.1 M Phosphate buffer (pH 6.0)
= 10 / 10 / 80

Asp

Cys

Ser
Labeling Reagent for Fluorescence Detection of Amines and Thiols

DBD-F
[=4-(N,N-Dimethylaminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole]

The compound A5595 which is an HPLC fluorescence labeling reagent having a 2,1,3-benzoxadiazole skeleton, can easily react with amino and mercapto groups to form the corresponding derivatives. The derivatives are stable enough to reach the detector without any decomposition under reversed phase HPLC. An excellent chromatogram can be obtained by fluorescence detection at the excitation and emission wavelengths of 450 nm and 590 nm, respectively.

Application example:
[Amino acids]
0.5 mL of 20 mM labeling reagent A5595 in acetonitrile is put into an amber-glass vial. To this solution, add 0.5 mL of 0.1 M boric acid buffer solution (pH 9.3, containing 1mM EDTANa2) containing several nmol of an amino acid, and incubate at 50 °C for 30 min. After cooling the reaction mixture with ice bath, use it as an HPLC sample.
For example, the detection limit (S/N=3) for proline is 0.11 pmol.

References
Chromatogram of amino acids as DBD-amino acids

Column: Kaseisorb LC ODS-120-5
Column Size: 4.6 mm I.D. × 250 mm
Mobile Phase: CH₃CN / H₂O / CH₃COOH = 50 / 50 / 1
Detection: Fluorescence λex 450 nm λem 590 nm
Flow Rate: 1 mL / min
Sample: 1. Valine
2. Leucine
Labeling Reagent for LC-MS/MS Analysis of Proteins

DAABD-Cl
[=4-[2-(Dimethylamino)ethylaminosulfonyl]-7-chloro-2,1,3-benzoxadiazole]

![Chemical Structure of DAABD-Cl]

The relationship between genes and diseases has been studied extensively since the completion of the human genome project in 2003. The direct cause of these diseases is sometimes related to the proteins produced in the human body by the human genome. The study of these proteins, "proteomics", is very important in order to understand the relationship between genes and diseases.

The general method for protein analysis is isolation of the targeted protein by 2-D gel electrophoresis, followed by digestion with proteases to yield peptide fragment mixtures, which are then analyzed by MS/MS to identify the fragments, from which the isolated protein can then be reconstructed. However, several problems still remain with 2-D gel electrophoresis, as extremely acidic, basic, or hydrophobic proteins cannot be fully separated. Furthermore, only the highly skilled experts are able to manage the 2-D gel electrophoresis to obtain reproducible data. For these reasons, new and improved methods for protein analysis have been explored.

Imai and co-workers have developed a new method for protein analysis with use of DAABD-Cl (A5596). This new method can analyze proteins with high precision. Imai and co-workers extracted proteins from breast cancer cells, and the extracted proteins were first reacted with tris(2-carboxyethyl)phosphine in a buffer solution in order to reductively cleave the S-S bonds to yield the primary proteins. The resulting SH functional groups of resulting proteins were derivatized by reaction with DAABD-Cl to yield fluorescent labeled protein mixtures (2 in Scheme 1). The fluorescent labeled protein mixtures were separated by fluorescence HPLC to obtain fractions consisting of DAABD labeled proteins (Fig. 1). The selected DAABD labeled protein (3 in Scheme 1) was isolated and digested using trypsin to obtain the peptide mixtures (4 in Scheme 1) consisting of DAABD labeled peptides and other peptides. 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 (Scheme 1).

Scheme 1. Quantification and Identification of Expressed Proteins in cell with DAABD-Cl

A-1094E
Scheme 1. Quantification and Identification of Expressed Proteins in cell with DAABD-Cl

Metastatic breast cancer cell

Galectin-1 which is increased expression in metastatic breast cancer cell compared to the non-metastatic cells

Fig. 1. Chromatograms of the proteins in soluble fraction of breast cancer cells derivatized with DAABD-Cl

The chlorine at 7 position of DAABD-Cl reacts specifically with SH groups. DAABD-Cl itself is non-fluorescent, however the resultant DAABD-derivative is strongly fluorescent, due to the benzoxadiazole skeleton coupled to the SH group. Generally, there are not many S-S bonds and SH group in proteins, and consequently target proteins can be labeled with DAABD-Cl in an efficient manner. Additionally, both excitation and emission wavelengths of DAABD derivatives are long, allowing highly sensitive and selective protein analysis. Furthermore, DAABD-Cl has a dimethylamino group at 4 position, and therefore high intensity cations can be obtained with electron spray ionization during MS analysis. Therefore, extremely small quantities of peptides can be analyzed.

DAABD-Cl is a labeling reagent, which can effectively permit the collection of the target protein through fluorescence HPLC and analysis by MS/MS. This protein analysis reagent that Imai and co-worker have developed allows one to identify a very small amount of protein with good precision. It is expected that this technique (FD-LC-MS/MS method) can be used in many applications, including the identification of abnormal or pathogenic proteins in living organism.

References
7) T. Ichibangase, K. Imai, J. Proteome Res. 2009, 8, 2129.
## TCI product number list

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HPLC Labeling Reagents