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An Alkylated Dihydroxybenzoic Acid Derivative as a Novel MALDI Matrix Additive for Sensitive Detection of Hydrophobic Peptides

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1: Overview.

- Hydrophobic peptides are generally difficult to detect using matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) because the majority of MALDI matrices are hydrophilic and therefore have a low affinity for hydrophobic peptides.
- Here, we report on a novel matrix additive, alkylated dihydroxybenzoic acid (ADHB), which is a 2,5-dihydroxybenzoic acid (DHB) derivative incorporating a hydrophobic alkyl chain on a hydroxyl group to improve its affinity for hydrophobic peptides, thereby improving MALDI-MS sensitivity.
- The addition of ADHB to the conventional matrix α-cyano-4-hydroxycinnamic acid (CHCA) improved the sensitivity of hydrophobic peptides 10- to 100-fold. The sequence coverage of phosphorylase b digest was increased using ADHB.
- MS imaging indicated that hydrophobic peptides were enriched in the rim of a matrix/analyte dried spot when using ADHB.
- In conclusion, the addition of ADHB to the standard matrix led to improved sensitivity of hydrophobic peptides by MALDI-MS.

2: Introduction.

- MALDI-MS is the most suitable for analyzing hydrophilic peptides, because conventional MALDI matrices have hydrophilic properties and thus have a low affinity for hydrophobic peptides. The limited solubility of hydrophobic peptides in aqueous solvent is alsoproblematic.
- Here, a novel matrix additive, alkylated dihydroxybenzoic acid (ADHB), was synthesized for highly sensitive analysis of hydrophobic peptides. ADHB is a 2,5-dihydroxybenzoic acid (DHB) derivative incorporating a hydrophobic alkyl chain on the hydroxyl group and thus is expected to have affinity for hydrophobic peptides.

3: Methods.

3-1: Peptides and Proteins.

- The peptides and digests were dissolved in 50/50 acetonitrile (ACN)/0.1% aqueous trifluoroacetic acid (TFA) (v/v) at appropriate concentrations.

3-2: Matrices.

- α–Cyano-4-hydroxycinnamic acid (CHCA) was purchased from LaserBio Labs.
- Each matrix was dissolved in 50/50 ACN/0.1% aqueous TFA (v/v) at 10 mg/mL.

3-3: 2-Hydroxy-5-octyloxybenzoic acid (ADHB).

- ADHB incorporating a C8 alkyl chain (**Figure 1**) was synthesized.
- ADHB solution was prepared in 50/50 (v/v) ACN/0.1% aqueous TFA at 5 mg/mL.

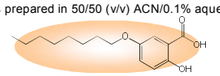


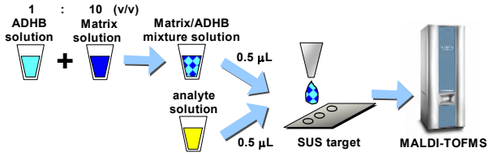
Figure 1. ADHB as a novel matrix additive

3-4: Sample preparation.

- ADHB solution and matrix solution were mixed at ratios of 1:10 (v/v). The analyte solution (0.5 μL) and the matrix solution containing ADHB (0.5 μL) were mixed on a stainless-steel plate to be analyzed by MALDI-TOFMS (**Scheme 1**).

3-5: MALDI-MS.

- MALDI-TOFMS measurement was performed using *AXIMA Performance*™ (Shimadzu/Kratos, UK) mass spectrometer in linear mode, in positive and negative ion modes.

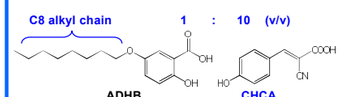


Scheme 1. Sample preparation using ADHB.

4: Results.

4-1: Optimization of ADHB Usage.

● **Optimized condition of ADHB for humanin (Scheme 2):**
- Alkyl chain lengths : the octyl (C8) chain (**Table 1**).
- Matrix: CHCA, compared to DHB, 3,5-dimethoxy-4-hydroxycinnamic acid, or 1,5-diaminonaphthalene.
- Mixture ratio: 1:10 (v/v), in the comparison of the 1:1, 10:1, 100:1, and 1:10 (v/v) mixtures of CHCA solution and ADHB solution.



Scheme 2. Optimized ADHB condition.

Table 1. Detection limit of hydrophobic peptide humanin[a]

	humanin (fmol/well)	
	positive mode	negative mode
C16-ADHB+CHCA	1	10
C10-ADHB+CHCA	1	10
C8-ADHB+CHCA	0.1	1
C6-ADHB+CHCA	1	10
C4-ADHB+CHCA	10	100
C1-ADHB+CHCA	10	100
CHCA	10	100

[a] Detection limit of humanin using CHCA with ADHB possessing different alkyl chain lengths of C1, C4, C6, C8, C10, or C16 as CHCA+ADHB or CHCA alone.

● ADHB incorporating octyl chain (C8-ADHB), the abbreviation "ADHB", and the 1:10 (v/v) mixture with CHCA was used in all the experiments.

4-2: Analysis of Hydrophobic and Hydrophilic Peptide Mixture.

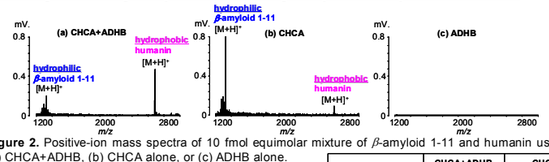


Figure 2. Positive-ion mass spectra of 10 fmol equimolar mixture of β-amyloid 1-11 and humanin using (a) CHCA+ADHB, (b) CHCA alone, or (c) ADHB alone.

● **Analysis of hydrophobic and hydrophilic peptide mixture:**
- Humanin was detected with higher intensity than β-amyloid 1-11 using CHCA+ADHB, whereas β-amyloid 1-11 was detected with higher intensity using CHCA alone (**Figure 2**).
- Neither peptides were detected using ADHB alone.

➡ ADHB contributes to an intensity increase of hydrophobic peptide ions.

● **MS images of hydrophobic and hydrophilic peptides:**
- With the use of CHCA+ADHB, humanin was detected mainly in the rim of the matrix/analyte spot, and β-amyloid 1-11 was detected throughout the entire spot, with the exception of the rim (**Figure 3**). With the use of CHCA alone, these peptides were detected throughout the entire spot.
- ADHB itself was detected mainly in the rim of the dried spot, and CHCA itself was detected throughout the entire spot.

➡ ADHB enriches hydrophobic peptides.

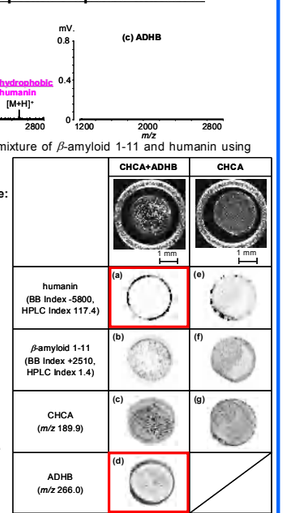


Figure 3. Matrix/analyte crystal photos (top rows) on a sample plate and MS images of (a, e) humanin, (b, f) β-amyloid 1-11, (c, g) CHCA and (d) ADHB for a 10 fmol equimolar mixture of humanin and β-amyloid 1-11 using CHCA+ADHB or CHCA alone, using the raster scanned datasets. The same dataset as for Figure 2 was used.

4-3: Sensitivity Improvement and Peptide Hydrophobicity.

● **BB Index and Sensitivity improvement for 14 peptides using ADHB:**

- **Table 2** based on the BB Index indicated sensitivity Improvement using ADHB for analytes No. 1-7 (BB Index -9210 ~ -1760) and No. 10.

➡ Sensitivity improvement with ADHB was related to peptide hydrophobicity as indicated with the BB Index, with an exception.

- Improvement in sensitivity using ADHB was observed for peptides with a molecular weight (M.W.) exceeding 2000, except for analyte Nos. 1, 9, and 12.

➡ M.W. or the surface area of a peptide may be related to sensitivity improvement.

Table 2. Sensitivity improvement for peptides with the BB Index from -9210 to +9030[a]

No.	BB Index	M.W.	name	detection limit (fmol/well) CHCA+ADHB	detection limit (fmol/well) CHCA	sensitivity improvement rate (fold)[b]
1	-9210	1396.8	temporin A, amide	0.1	1	10
2	-7070	2781.5	NF-kB inhibitor	0.1	10	100
3	-5800	2687.2	humanin	0.1	10	100
4	-5410	2657.2	[Gly14]-humanin	0.1	1	10
5	-5130	2290.5	OVA-BIP hybrid peptide	0.1	10	100
6	-4470	2846.5	melittin, honey bee	0.1	1	10
7	-1760	4514.1	β-amyloid 1-42	0.1	10	100
8	-1500	1999.3	β-amyloid 22-42	1	1	1
9	-380	2465.7	ACTH 18-39	0.1	0.1	1
10	+310	2766.2	MPGΔNLS	0.1	1	10
11	+2510	1325.3	β-amyloid 1-11	1	1	1
12	+2690	3262.5	β-amyloid 1-28	0.1	0.1	1
13	+4650	1364.5	GPHRSTPESRAAV	1	1	1
14	+9030	1847.8	β-conglycinin 165-178	0.1	0.1	1

[a] Detection limit and sensitivity improvement for 14 peptides with a BB Index from -9210 to +9030 using CHCA+ADHB, compared with CHCA alone in the positive-ion mode.
[b] Sensitivity improvement was calculated by dividing the detection limit using CHCA alone by that using CHCA+ADHB.

● **HPLC Index and Sensitivity improvement of 14 peptides using ADHB:**

◆ The HPLC Index is a hydrophobicity scale based on predictive retention coefficients of peptides by RP-HPLC. This index indicates the interaction between the peptide and the C18 alkyl chain attached to the stationary phase of a reverse-phase column.

- **Table 3** based on the HPLC Index indicated sensitivity improvement using ADHB for hydrophobic peptides with an HPLC Index of 100 or higher.
- These peptides were all detected in the rim of the matrix/analyte spot.

➡ Sensitivity improvement using ADHB was more closely related to the HPLC Index than the BB Index. It may be due to interactions between peptides and the alkyl chain of ADHB.

Table 3. Sensitivity improvement, HPLC Index and MS images of peptides[a]

No.	HPLC Index	name	sensitivity improvement rate (fold)[b]	MS imaging[c] CHCA+ADHB	MS imaging[c] CHCA
2	200.0	NF-kB inhibitor	100		
10	125.6	MPGΔNLS	10		
4	120.3	[Gly14]-humanin	10		
6	117.8	melittin, honey bee	10		
3	117.4	humanin	100		
1	110.9	temporin A, amide	10		
7	110.4	β-amyloid 1-42	100		
5	100.8	OVA-BIP hybrid peptide	100		
9	58.9	ACTH 18-39	1		
12	44.7	β-amyloid 1-28	1		
8	44.5	β-amyloid 22-42	1		
13	3.3	GPHRSTPESRAAV	1		
11	1.4	β-amyloid 1-11	1		
14	-60.2	β-conglycinin 165-178	1		

[a] Sensitivity improvement of 14 peptides using CHCA+ADHB in positive-ion mode. The number on the left-hand side of the table is the same as in Table 2.
[b] Sensitivity improvement was calculated by dividing the detection limit using CHCA alone by that using CHCA+ADHB for differentially-analyzed peptides.
[c] MS images were created for each peptide in the 100 fmol 14 peptide mixture analyses.

4-4: Analysis of Phosphorylase b Digests.

● **Improvement of sequence coverage of phosphorylase b using ADHB:**

- In the *m/z* >2500 range, ions were observed more strongly using ADHB, especially for ions at *m/z* 3715.2 and *m/z* 4899.3, with a relatively high HPLC Index (**Figure 4**).
- The two peptides were detected in the rim.

➡ Sequence coverage increased from 53.9% (CHCA alone) to 63.2% (CHCA+ADHB)(10% increase).

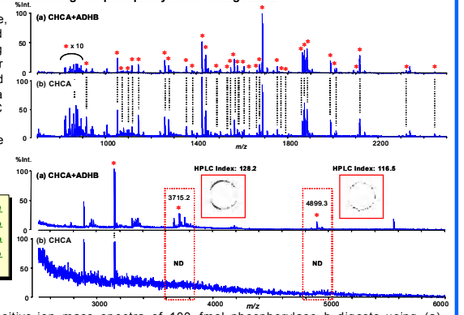


Figure 4. Positive-ion mass spectra of 100 fmol phosphorylase b digests using (a) CHCA+ADHB or (b) CHCA alone in *m/z* 700 to 2500 (top) and *m/z* 2500 to 6000 (bottom). The digest ion peaks are annotated with asterisks. ND indicates that ion peaks were not detected. Insets are MS images of ion peaks at *m/z* 3715.2 and *m/z* 4899.3 using CHCA+ADHB.

4-5: Peak Intensity Distribution of Hydrophobic Peptides.

● **Mass spectra and heat maps of $[M+H]^+$ or $[M+Na]^+$:**

- MS images demonstrates that $[M+H]^+$ of the peptide was strongly detected in the rim of a matrix/analyte spot using CHCA+ADHB (**Figure 5**).
- Using CHCA alone, the peptide was detected as $[M+H]^+$ and $[M+Na]^+$ in the whole area of the spot with a relatively lower intensity.
- The lower signal of sodiated ions observed using CHCA+ADHB.

➡ Hydrophobic peptides were predominantly detected as $[M+H]^+$ with small amounts of sodium salts using CHCA+ADHB (desalting effect).

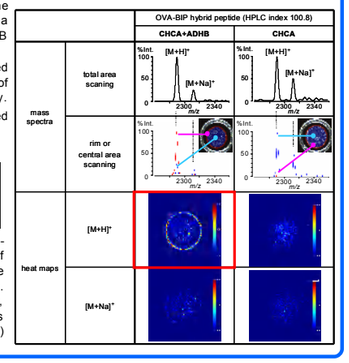
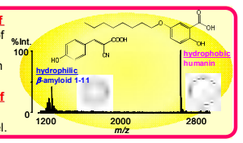


Figure 5. Mass spectra and heat maps color-coded according to absolute peak intensity of $[M+H]^+$ or $[M+Na]^+$ for the hydrophobic peptide OVA-BIP using CHCA+ADHB or CHCA alone. Mass spectra for the entire surface (top row), and the second row represents each mass spectrum accumulated along the rim (red trace) or in the entire spot (blue trace).

5: Conclusions.

- A novel matrix additive ADHB improved the sensitivity of hydrophobic peptides through their enrichment in the rim of a matrix/analyte dried spot.
- The protocol is simple: CHCA is mixed with ADHB and then used as the matrix.
- The addition of ADHB to CHCA improved the sensitivity of hydrophobic peptide 10- to 100-fold.
- Hydrophobic peptides were detected at the subfemtomole level.



References

- Fukuyama, Y.; Tanimura, R.; Maeda, K.; Watanabe, M.; Kawabata, S.;Iwamoto, S.; Izumi, S.; Tanaka, K. *Anal. Chem.* **2012**, *84*, 4237-4243.

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