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Deuterated Matrix-Assisted Laser Desorption Ionization Matrix Uncovers Masked Mass Spectrometry Imaging Signals of Small Molecules

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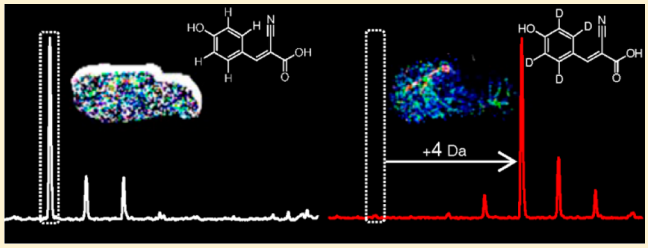
Supporting Information

ABSTRACT: D^4-α-Cyano-4-hydroxycinnamic acid (D^4-CHCA) has been synthesized for use as a matrix for matrix-assisted laser desorption ionization-mass spectrometry (MALDI-MS) and MALDI-MS imaging (MSI) of small molecule drugs and endogenous compounds. MALDI-MS analysis of small molecules has historically been hindered by interference from matrix ion clusters and fragment peaks that mask signals of low molecular weight compounds of interest. By using D^4-CHCA, the cluster and fragment peaks of CHCA, the most common matrix for analysis of small molecules, are shifted by + 4, + 8 and + 12 Da, which expose signals across areas of the previously concealed low mass range. Here, obscured MALDI-MS signals of a synthetic small molecule pharmaceutical, a naturally occurring isoquinoline alkaloid, and endogenous compounds including the neurotransmitter acetylcholine have been unmasked and imaged directly from biological tissue sections.



Matrix-assisted laser desorption ionization (MALDI) mass spectrometry (MS) is a soft ionization technique which, when coupled to one of a broad range of mass analyzers, enables high sensitivity analyte detection and is used across a diverse variety of research areas.[1] Since the development of the technique by Hillenkamp and Karas,[2] it has been utilized for the analysis of biological macromolecules (peptides,[3] proteins, and protein complexes[4]), synthetic polymers,[5] and even nanoparticles.[6] MALDI-MS imaging (MALDI-MSI) is an application of the technology that enables label-free compound distribution mapping of molecular species directly from tissue sections.[7] By performing MALDI-MSI analysis directly on the surface of a tissue section, the technique has quickly been established as a powerful in situ visualization tool for measuring relative abundance and spatial distribution of endogenous and pharmaceutical small molecule compounds, lipids, and proteins.[8,9]

MALDI works through the cocrystallization of the MALDI matrix, an organic compound, with analytes in the sample. The matrix is able to absorb the laser energy pulse (typically at a wavelength of 355 or 337 nm) which then causes the subsequent desorption and ionization of the analyte and matrix. However, during the ionization process the matrix generates fragment and cluster ions which are low in molecular weight (typically <600 Da). These ions can significantly mask the signal of any analytes with a similar mass-to-charge (m/z) ratio, hence limiting the ability to analyze some endogenous small molecules, pharmaceutical compounds, and metabolites.[10]

To overcome the problem, several strategies can be attempted. The simplest is to switch to an alternative MALDI matrix which will generate a different set of matrix cluster ions. However, different MALDI matrices have different ionization properties and will often not enable sufficient ionization of the target analyte. More complex approaches have been developed which includes desorption ionization from porous silicon (DIOS),[11] nanostructure initiator mass spectrometry (NIMS),[12] high molecular weight matrices,[13] and surface-assisted laser desorption ionization.[14] Despite some success, these methods have never been as widely used as MALDI because of the technical and handling difficulties for conventional MS and especially MS imaging.

Following the development of α-cyano-4-hydroxycinnamic acid (CHCA),[15] it has become the most commonly used

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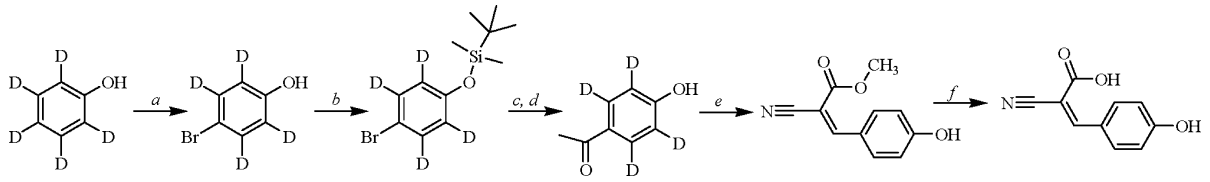
Scheme 1. Synthesis of D^4-CHCA[a]



[a](a) Br_2/dioxane, 0 °C; (b) TBDMSCl/DMF, imidazole/DMF, RT; (c) *n*-BuLi, THF, N_2, −65°C, DMF then aq HCl, 0 °C; (d) methanol/HCl, N_2, RT; (e) cyanoacetic acid ethyl ester, piperidine, toluene, 5h; (f) LiOH, RT, 2 h, water then aq HCl.

MALDI matrix for both spotted sample and tissue imaging MALDI analysis and it is very effective for the ionization of small molecules. However, there are a large number of cluster peaks detected <600 Da, causing complications in analyte identification or quantitation. By synthesizing D^4-CHCA that retains the same physicochemical properties of the standard CHCA (the acidity and light absorption properties) means that the *m*/*z* ratios of matrix cluster and fragments peaks are shifted. Therefore, by alternating between D^4-CHCA and standard CHCA the entire lower mass range can be analyzed without interference of the matrix cluster, while retaining the effective ionization properties of the CHCA matrix.

EXPERIMENTAL SECTION

Chemicals and Reagents. All chemicals and reagents were purchased from Sigma Aldrich (St. Louis, MO) unless otherwise noted and were used without further purification. Phenol-2,3,4,5,6-d_5 (D^5-phenol) was purchased from CDN Isotopes Inc. (Essex, U.K.). Water, methanol, trifluoroacetic acid (TFA), and TLC plates were obtained from Merck (Darmstadt, Germany).

Synthesis of D^4-CHCA. D^4-CHCA was synthesized using phenol-2,3,4,5,6-d_5 (D^5-phenol) as the starting material as shown in Scheme 1. In short, D^5-phenol was brominated, and the product was then protected by *tert*-butyldimethylsilyl (TBDMS) in dimethylformamide (DMF) at ambient temperature in the presence of imidazole. The product was purified by distillation under reduced pressure. 4-Hydroxybenzaldehyde was synthesized in tetrahydrofuran (THF) at −65 °C using *n*-butyllithium (*n*-BuLi) and DMF. 4-Hydroxycinnamic ester was prepared using methyl cyanoacetate in the presence of piperidine in toluene. The final product was obtained by hydrolysis with lithium hydroxide (LiOH) followed by crystallization from 2-propanol. The detailed synthesis procedure is described in the Supporting Information.

Animal Experiments and Tissue Preparation. Animal experiments were conducted in accordance with the European Communities Council Directive of November 24, 1986 (86/609/EEC) approved by the Ethical Committees on Animal Experiments in Lund/Malmö, Sweden (no. M84-05) and at Karolinska Institute, Stockholm, Sweden (no. N351/08). Adult male wistar rats weighing approximately 350 g were used. The animals were kept in a climate controlled facility (22 ± 3 °C, 55 ± 10% relative humidity) with 12 h light−dark cycles. Food and water were given ad libitum. Amiloride was administered (800 μg/kg) through intratracheal injection. Control and dosed animals were anesthetized and sacrificed 2 h post administration. The lung and brain were rapidly dissected out and immediately placed in a fulminating bath of isopentane/dry ice on a hard plastic dish. The samples were stored at −80 °C until required for sectioning.

The C57Bl/6 male mouse, 3 months old, was housed in air-conditioned rooms (12 h dark−light cycles) at 20 °C with a humidity of 53%. The mouse was euthanized, and the brain was rapidly removed, frozen in dry ice-cooled isopentane, and stored at −80 °C.

The frozen lung and brain tissues were cut by a cryostat-microtome (Leica CM3050S, Leica Microsystems, Germany). Lung sections were taken from the long flat frontal plane of the left lobe, providing sections of the central airways at the thickness of 12 μm. Control lung tissue sections and a lung tissue section from a dosed animal were placed on the same MALDI target glass slide. Sagittal brain sections were cut at a thickness of 12 μm. Tissue sections were transferred by thaw mounting onto conductive indium tin oxide (ITO) glass slides (Bruker Daltonics, Bremen, Germany), prior to storage at −80 °C. Sections were desiccated at room temperature for 15 min prior to MALDI matrix application. Tissue sections were not washed prior to matrix application.

MALDI-MS and MALDI-MSI Analysis. For MALDI-MS analyses, 0.5 μL of matrices, (CHCA and D^4-CHCA, 7 mg/mL, 50% acetonitrile, 0.2% TFA) were spotted and dried on a stainless steel MALDI plate (Bruker Daltonics). Berberine (0.5 μL of 2 pmol/μL in 50% acetonitrile, 0.2% TFA) was spotted on dried spots of CHCA and D^4-CHCA. Collected spectra were summed from 500 laser shots (355 nm Nd:YAG Smartbeam laser, Ultraflex II MALDI time-of-flight (TOF)/TOF mass spectrometer, Bruker Daltonics, Bremen, Germany).

For MALDI-MS imaging analyses the MALDI matrices (CHCA and D^4-CHCA, 7 mg/mL in 50% acetonitrile, 0.2% TFA) were applied to the MALDI glass targets using an automatic matrix sprayer (ImagePrep, Bruker Daltonics). MALDI-MS and MALDI-MSI analyses were performed using an Ultraflex II TOF/TOF mass spectrometer (Bruker Daltonics) in positive ion reflectron mode using a Smartbeam II 200 Hz laser. The mass spectrometer parameters were as manufacturer's recommended settings adjusted for optimal acquisition performance. The laser spot size was set at medium focus (~50 μm laser spot diameter), and laser power was optimized at the start of each run and then fixed for the MALDI-MSI experiment. Tissue sections were analyzed in a random order to prevent any possible bias due to such factors as matrix degradation or variation in mass spectrometer sensitivity. MSI data was analyzed and normalized using FlexImaging, version 2.0 (Bruker Daltonics). Regions of interest were manually defined in the analysis software using both the optical image and MSI data image. Masses were selected with a mass precision of ±0.1 Da.

RESULTS AND DISCUSSION

The study reported here describes the synthesis and use of D^4-CHCA for the detection of pharmaceutical and endogenous compounds that could not previously be detected due to

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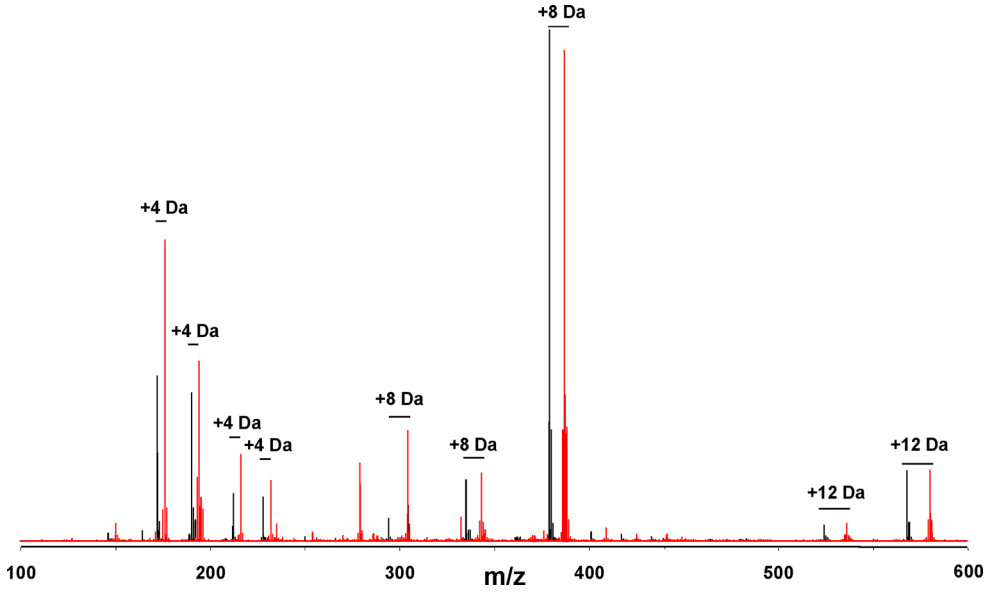

Figure 1. MALDI mass spectra of CHCA (black) and D^4-CHCA (red). The fragment/cluster peaks in the low mass range are shifted by + 4, + 8, and + 12 Da when D^4-CHCA is used as an alternative matrix to uncover the hidden m/z ratios.

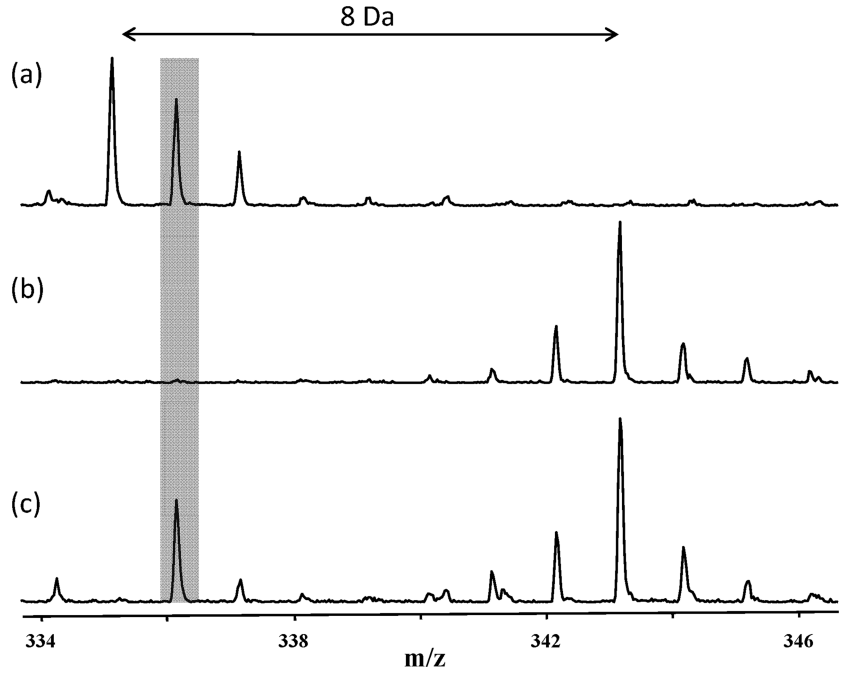

Figure 2. (a) MALDI mass spectra of CHCA, (b) D^4-CHCA, and (c) berberine acquired by D^4-CHCA as matrix. The overlapping peak of CHCA at 336.1 Da masks the signal of berberine while the clean background of D^4-CHCA in this region facilitates uncovering the berberine signal.

interference by CHCA matrix cluster peaks. D^4-CHCA is used for the analysis of both spotted sample and tissue sample analysis.

Following the synthesis of D^4-CHCA matrix, the physicochemical properties of the product were analyzed to confirm that no detrimental effect had occurred. The D^4-CHCA produced was found to have the same light absorption pattern as standard CHCA as shown in the Supporting Information (Figure S1). Initial comparison between CHCA and D^4-CHCA was made by spotting of each matrix onto a stainless steel MALDI target and collecting spectra as described in the Experimental Section. The CHCA clusters and fragments were shifted by + 4, + 8, and + 12 Da (Figure 1).

More than 50 fragment/cluster peaks of CHCA in the low molecular weight region (100−600 Da) of the mass spectra were found to be shifted when using the deuterated matrix. A ChemSpider chemical database search (Royal Society of Chemistry, Cambridge, U.K.) resulted in approximately 19 million known compounds in the target mass region (100−600 Da), and of that 19 million, approximately 1.5 million would potentially be masked by the interfering fragments/clusters of CHCA given the centroid masses of fragment/cluster peaks of ±0.1 Da (typical of mass analyzers such as quadrupole, ion trap, and time-of-flight). Therefore, by shifting fragment/cluster peaks it can be anticipated that a significant number of endogenous and synthetic compounds may well be detected

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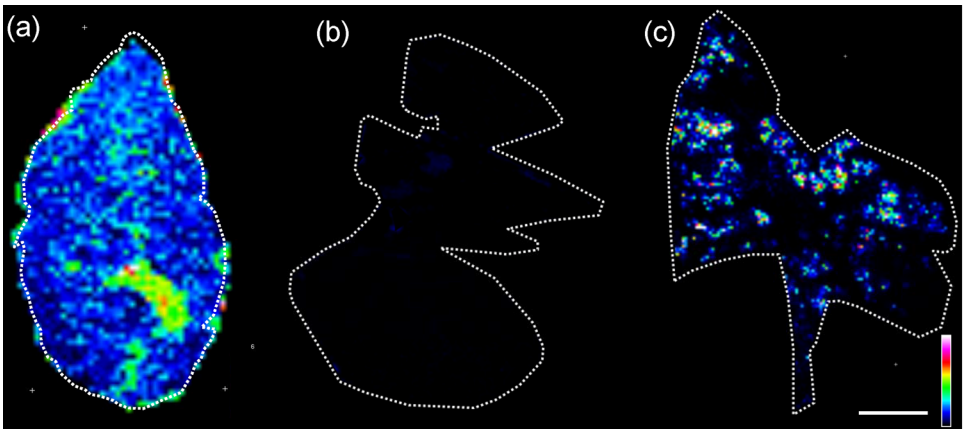

Figure 3. MALDI-MSI relative abundance and distribution of m/z 230.0 ± 0.1 in rat lung tissue sections. (a) The cluster product of CHCA at m/z 230.0 is observed on the control tissue covered by CHCA as the matrix. (b) No signal at m/z 230.0 is observed on the control tissue when D^4-CHCA is used as the matrix, which facilitates an appropriate condition for imaging of amiloride. (c) The spatial distribution of amiloride is imaged in a rat lung tissue section of an in vivo amiloride administered rat using D^4-CHCA as the matrix. Difficulty in sectioning lung tissue produced samples with different morphology. (Data displayed in the rainbow scale over the same range, scale bar 2 mm, and the dashed line marks the outline of the tissue section).

when using D^4-CHCA as the MALDI matrix. Absolute intensity values are not always reliable during MALDI analysis. Different factors such as matrix crystal structures and the amount of salts in the sampling spots can affect the detected abundance of target compounds. However, from Figure 1 it is clear that D^4-CHCA gives a higher detected abundance for molecular ions and fragments while the signal abundance for matrix clusters are almost the same. This could be attributed to C−D, C−H bond energy values and gas phase reactivity differences.

Berberine (m/z 336.1), a naturally occurring potent therapeutic isoquinoline alkaloid,[16] is an example of a small molecule pharmaceutical compound which is masked from detection by MALDI by a matrix cluster of CHCA at m/z 336.1, as demonstrated in Figure 2. The use of D^4-CHCA shifts the matrix cluster by +8 Da and allows the unmasked detection of berberine. Therefore, by spotting a sample twice, once with standard and once with D^4-CHCA, it is possible to mitigate the risk of matrix cluster interference and unfettered access to the whole mass spectrum is obtained.

D^4-CHCA can also be demonstrated to provide a means to analyze small molecule synthetic pharmaceutical compounds that have m/z ratios that are masked by the standard CHCA matrix. Amiloride is an antikaliuretic-diuretic pharmaceutical used for management of congestive heart failure and hypertension. The third isotopic peak of a CHCA cluster overlaps with protonated amiloride (m/z 230.0) and hence prohibits its detection and distribution to be determined by MALDI analyses. Following the administration of amiloride to rats, tissue sections were coated in either standard or D^4-CHCA prior to MALDI-MSI analysis. Figure 3a shows the distribution of m/z 230.0 across a drug treated lung tissue section coated by standard CHCA. Because of the overlapping between the matrix cluster and the amiloride peak, no discernible distribution can be observed. However, when D^4-CHCA is applied to the control lung tissue section, no matrix cluster peak is detected at m/z 230.0 (Figure 3b) but in drug treated lung tissue the distribution of protonated amiloride (m/z 230.0) is clearly detected without the interference of any matrix cluster peaks (Figure 3c). We submitted amiloride ions to MS/MS analysis directly from tissue sections in an attempt to acquire a specific identification of the drug, but we were unable to obtain acceptable MS/MS spectra and identification was based on the corresponding m/z ratio.

The suitability of D^4-CHCA for use in MALDI-MSI detection and the mapping of the relative abundance of small molecule endogenous compounds across tissue sections was examined and shown to be an effective strategy in enabling the detection of compounds that were previously obscured by standard CHCA matrix cluster and fragment peaks. An example of how the use of D^4-CHCA can allow a previously masked endogenous compound in a mouse brain section to be detected is given in Figure 4. When the distribution of m/z 250.1 is mapped using the standard CHCA matrix, the strong signal obtained from the matrix cluster masks any possible detection of endogenous compounds with closely related masses (Figure 4a,c). However, when using D^4-CHCA the matrix cluster peak is shifted by +4 Da enabling detection of endogenous compounds at m/z 250.1 (Figure 4b,c). Subsequent MS/MS fragmentation of m/z 250.1 generated product ions of m/z 184 and m/z 136 that may indicate a phosphocholine functional group.

Acetylcholine (ACh) is an endogenous neurotransmitter that plays important roles in the peripheral and central nervous system[17] but has a mass (m/z 146.1) preventing MALDI-MSI analysis with CHCA. When the abundance and distribution of m/z 146.1 is mapped across a tissue section coated by standard CHCA matrix, the strong signal obtained from the matrix fragment masks any possible mapping of ACh. However, when using D^4-CHCA as the matrix the fragment peak is shifted by +4 Da meaning that any detected signal is as a result of endogenous compounds. The resulting distribution map shows the molecular distribution of ACh in brain tissue sections (Figure 5). The confirmation that m/z 146.1 is ACh was achieved by on-tissue tandem MS (MS/MS) fragmentation of m/z 146.1 (Figure S2 in the Supporting Information), where product ions of m/z 87.0 and m/z 104.1 are generated. The MALDI-MSI data shows that ACh is most abundant in brain structures such as the cerebral cortex, corpus callosum, ventral hippocampal commissure, thalamus, and cerebellum (Figure 5).

An alternative approach to overcome matrix cluster and fragment peak overlap would be the use of higher spectral resolution mass analyzers able to resolve the cluster and

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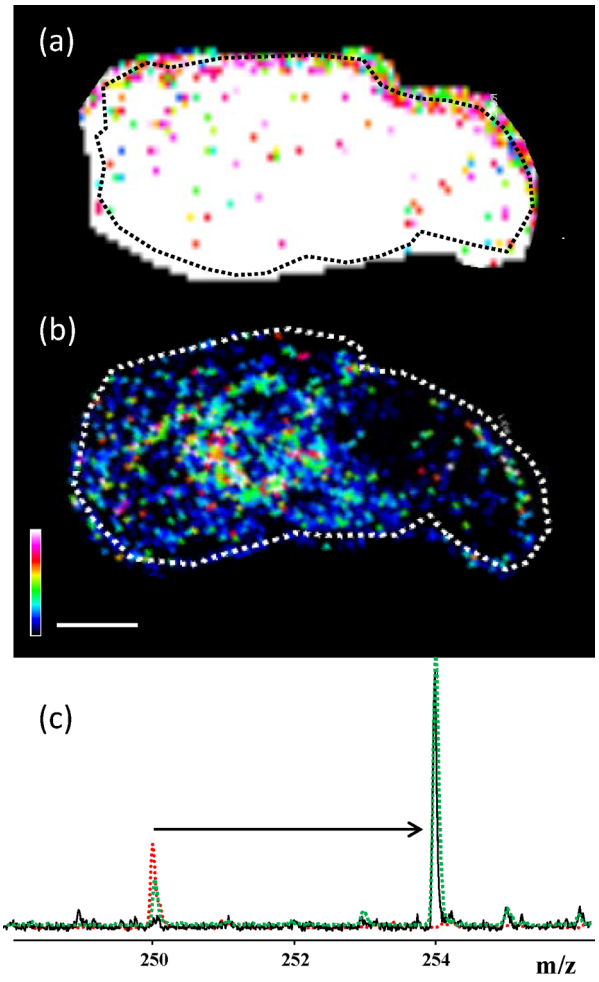

Figure 4. MALDI-MSI relative abundance and distribution of an endogenous compound with m/z 250.1 ± 0.1 in mouse brain tissue sections. (a) The cluster product of CHCA at m/z 250.1 is observed on the tissue covered by CHCA as the matrix. (b) The spatial distribution of the endogenous compound is imaged in a mouse brain tissue section using D^4-CHCA as the matrix. (c) MALDI mass spectra of CHCA (red-dashed line), D^4-CHCA (black-solid line), and the endogenous compound unmasked by D^4-CHCA (green-dashed line). (Data displayed in the rainbow scale over the same range, scale bar 2 mm, and the dashed line marks the outline of the tissue section). The overlapping peak of CHCA at m/z 250.1 masks the signal of the endogenous compound appearing in the same mass while the clean background of D^4-CHCA in this region facilitates uncovering and imaging of the endogenous compound.

compound masses. Attempts were made to detect ACh using a 12 T Fourier transform ion cyclotron resonance-mass spectrometer (FTICR-MS, Bruker Daltonics) equipped with the same type of MALDI laser; however, because of the lack of sensitivity toward the very low mass range region it was not possible to detect ACh. Hence, the use of D^4-CHCA is clearly demonstrated as a powerful addition to matrices used for the analysis of endogenous small molecule compounds.

CONCLUSIONS

In the data presented here we have shown that by using D^4-CHCA it is possible to perform MALDI-MS and MALDI-MSI of endogenous small molecule species such as the neurotransmitter acetylcholine as well as pharmaceutical compounds, with m/z ratios that meant they were previously obscured by

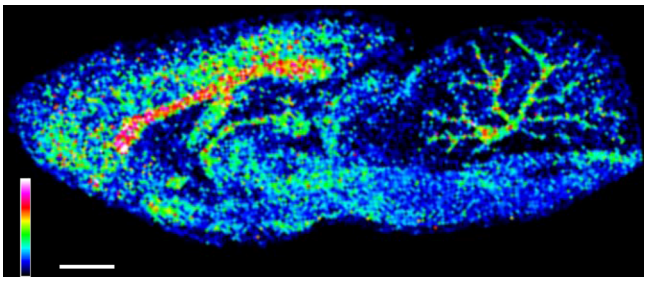

Figure 5. MALDI-MSI relative abundance and spatial distribution of the neurotransmitter acetylcholine (m/z 146.1 ± 0.1) in a rat brain sagittal tissue sections. The image corresponds to the tissue section covered by D^4-CHCA as the matrix. Unlike in Figures 2 and 4, a mass spectrum with no interference at m/z 146.1 is not presented for this example.

matrix cluster peaks when analyzed using standard CHCA. Through a combination of standard and D^4-CHCA matrices, researchers will be able to eliminate matrix interferences but still keeping the advantages of CHCA ionization efficiency for both MALDI-MS and MALDI-MSI analyses. It is believed that the matrix we have developed can be readily implemented in targeted and nontargeted MALDI-MS and MALDI-MSI workflows and will be of substantial benefit for researchers working within the pharmaceutical, metabolomics, and environmental fields. While an alternative method of overcoming analyte masking by overlapping matrix peaks could be the use of higher spectral resolution mass analyzers, there is often a lack of sensitivity in the low mass range where such masking occurs that makes detection of low abundance masked compounds impossible. There is also often limited availability of high-resolution instruments for MSI, compared to the more prolifically employed lower resolution instruments. Hence, the use of D^4-CHCA is clearly demonstrated as a powerful addition to matrices used for the analysis of small molecule compounds.

ASSOCIATED CONTENT

Supporting Information

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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