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RESEARCH ARTICLE

Cationic Xylene Tag for Increasing Sensitivity in Mass Spectrometry

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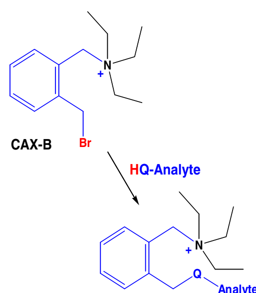

Abstract. *N*-(2-(Bromomethyl)benzyl)-*N*,*N*-diethylethanaminium bromide, that we designate as CAX-B (cationic xylyl-bromide), is presented as a derivatization reagent for increasing sensitivity in mass spectrometry. Because of its aryl bromomethyl moiety, CAX-B readily labels compounds having an active hydrogen. In part, a CAX-tagged analyte (CAX-analyte) can be very sensitive especially in a tandem mass spectrometer (both ESI and MALDI). This is because of facile formation of an analyte-characteristic first product ion (as a xylyl-based cation) from favorable loss of triethylamine as a neutral from the precursor ion. This loss is enhanced both by resonance stabilization of the xylyl cation, and by anchimeric assistance from the *ortho* hetero atom of the attached analyte. High intensity of a first product ion opens up the opportunity for a CAX-analyte to be additionally sensitive when it is prone to a secondary neutral loss from the analyte part. For example, we have derivatized and detected 160 amol of thymidine by CAX-tagging/LC-MALDI-TOF/TOF-MS in this way, where the two neutral losses are triethylamine and deoxyribose. Other analytes detected at the amol level as CAX derivatives (as diluted standards) include estradiol and some nucleobases. The tendency for analytes with multiple active hydrogens to label just once with CAX (an advantage) is illustrated by the conversion of bisphenol A to a single product even when excess CAX-B is present. A family of analogous reagents with a variety of reactivity groups is anticipated as a consequence of replacing the bromine atom of CAX-B with various functional groups.

Keywords: Derivatization, Mass tag, Cation, Mass spectrometry, DNA adducts, Metabolomics

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Introduction

A diversity of cationic mass tags have been studied for the purpose of enhancing the response of analytes in a mass spectrometer via derivatization. The cationic (or precationic) groups in these tags have been tertiary amines, including pyridine [1–5] (which forms a cation by protonation), quaternary amines (either pyridinium [6–12] or tetra-alkylammonium) [1, 9, 13–20], and phosphonium [20, 21]. The functional groups employed in these tags have been as follows: hydrazide [7, 13, 14, 16, 17], hydroxy [11, 12, 15], pyridine [6], acyl chloride [5, 16], bromomethyl [16, 18], thiocyanate [16], amino [1–3, 8], *o*-diamine [2], sulfonyl chloride [2], *N*-hydroxysuccinimide [18, 20, 21], anhydride [5], secondary amine [3], carboxylic acid [4, 9], fluoropyridinium [4], and 1,2,4-triazolone-3,5-dione [19]. Two kinds of mass spectrometers in terms of ion source mostly have been employed: ESI and MALDI.

Recent advances in this field include the detection of α,β-unsaturated ketones (bearing a ɣ-H) and alcohols (including carbohydrates and α,β-unsaturated alcohols) by derivatization with pyridine in the presence of trifluoromethanesulfonic anhydride [6]; tryptic peptides by derivatization with reagents having both a primary amine functional group and a tertiary or quaternary amine group (after prior capping of peptide primary amines by dimethylation) [1]; glycans by derivatization with hydrazide reagents bearing a pyridinium or phenylpyridinium group [7]; eicosanoids by derivatization with a pyridinium reagent having a benzylamine reactive group based on activation of the carboxyl group of the eicosanoids with a carbodiimide [8]; hydroxysteroids (after oxidation to ketones with cholesterol oxidase) by derivatization with Gerard's T reagent [17]; and chiral carboxylic acids after derivatization with chiral reagents having a tertiary amine, along with a primary or secondary amine as a reactivity group, in the presence of 4-(4,6-dimethoxy-1,3,5-triazin-2-

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yl)-4-methylmorpholinium chloride as a activating group for carboxyl [3].

Here we report a quaternary amine reagent having an aryl bromomethyl functional group, *N*-(2-(bromomethyl)benzyl)-*N*,*N*-diethylethanaminium bromide, which we designate as "CAX-B (cationic xylyl-bromide)", for the purpose of increasing the sensitivity of mass spectrometry for analytes having an active hydrogen. The representative analytes that we have detected to date with this reagent come from the classes of acids, alcohols, phenols, nucleosides, and nucleobases. In some cases, diluted standards of CAX-analytes were tested and found to be detectable at the amol level. Thymidine was both derivatized and detected at the amol level. Overall, on our equipment, the detection limits of the analytes studied here without tagging are at the high fmol to low pmol level.

Experimental

Materials

α,α′-Dibromo-*ortho*-xylene, α,α′-dibromo-*meta*-xylene, α,α′-dibromo-*para*-xylene, triethylamine, α-cyano-4-hydroxycinnamic acid (CCA), thymidine, nucleobases, Acyclovir, 2-phenylethanol, 1-butanol, 4-phenyl-1-butanol, deoxyribose, benzoic acid, bisphenol A, trifluoroacetic acid (TFA), and 17β-estradiol (E2) were from Sigma (St. Louis, MO, USA). *o*-Xylene-d_{10}, 99.3 atom% D, was from CDN Isotopes (Quebec, Canada). Bromine, 99.8%, was from Alfa Aesar (Ward Hill, MA, USA). The Phenol Calibration Mix (12 components, Cat. No. 31694) was from Restek (Bellefonte, PA, USA). Microcentrifuge tubes, pipette tips, ethyl acetate (certified ACS), and HPLC grade acetonitrile (ACN) were from Fisher Scientific (Pittsburgh, PA, USA). All materials were used as received. CCA matrix solution was 5 mg/mL in 50% ACN.

Instrumentation

The MALDI-TOF/TOF-MS was a model 5800 from AB-SCIEX (Framingham, MA, USA), as was the Eksigent Tempo LC MALDI system. The ion trap MS was a LTQ Velos Pro from Thermo Fisher (Tewksbury, MA, USA). The HPLC system and conditions for detection of CAX-thymidine, after labeling at the amol level, by HPLC/ion trap/MS were as follows. Thermo Scientific Ultimate 3000 RSLC; column: 2.1 × 50 mm, 1.9 μm Hypersil Gold C18 (Thermo Scientific); column temperature: 35°C; sample tray temperature: 10°C; mobile phase: [A] H_2O with 0.1% formic acid, [B] ACN with 0.1% formic acid; gradient: 10%–90% B in 2 min, hold for 0.5 min, re-equilibrate for 0.5 min; flow rate 0.5 mL/min; injection volume 5 μL. The capillary LC (CapLC) system for detection of CAX-analytes by CapLC-MALDI-TOF/TOF was as follows. Dionex Ultimate (Thermo Scientific); column: 0.3 × 150 mm, 2 μm Acclaim PepMed C18 (Thermo Scientific).

Synthesis

N-(2-(Bromomethyl)benzyl)-N,N-diethylethanaminium bromide, that we designate as CAX-B α,α′-Dibromo-*o*-xylene (200 mg, 0.76 mmol) was dissolved in 1 mL ACN and Et_3N (0.122 mL, 0.87 mmol) was added, yielding a white precipitate after several min. The mixture was stirred at room temperature for 6 h and then stored at –20°C overnight. Pipetting off the upper layer of solvent and washing the residue with cold ACN (2 × 2 mL) with intermittent settling gave 156 mg (56%) of product as a white solid.

1,2-Bis(Bromomethyl)Benzene-d_8 Similar to a prior method [22], into a two-necked 25 mL rb flask was added 0.5 mL (4.10 mmol) of *o*-xylene-d_{10} with 10 mL of water. Under an inert N_2 atmosphere, 0.21 mL (4.10 mmol) of Br_2 was added dropwise over 5 min. The mixture was then irradiated with a 300 W halogen bulb about 10 cm away for about 30 min (until the orange color faded to light yellow or clear). After cooling to room temperature, extraction was done with 3 × 10 mL of EtOAc followed by Na_2SO_4 drying of the combined extracts and then evaporation under reduced pressure, yielding 280 mg (25%) of product as a white solid.

N-[2-(Bromomethyl)benzyl]-N,N-Diethylethanaminium-d_8 Bromide (CAX-B-d_8) Similarly to above, 86 mg (0.32 mmol) of 1,2-*bis*(bromomethyl)benzene-d_8 in 0.5 mL of ACN along with 0.05 mL (0.36 mmol) of Et_3N yielded 61 mg (52%) of product as a white solid.

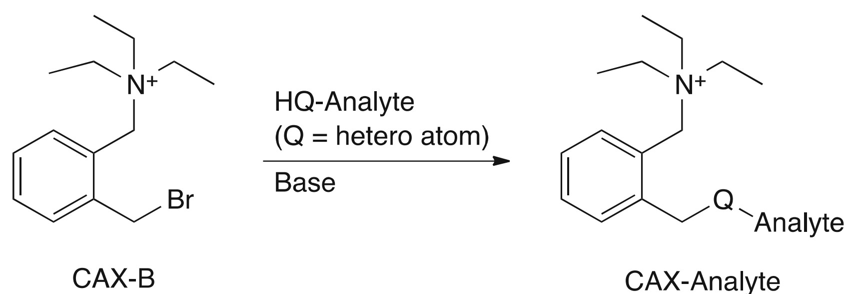

Figure 1. Reaction scheme for tagging an analyte having an active hydrogen with CAX-B

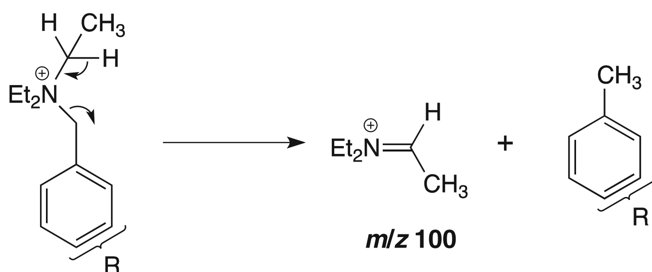

Figure 2. Scheme for rationalizing the relatively intense formation of *m/z* 100 from CAX-estradiol in MALDI-TOF/TOF-MS when the *meta* or *para* isomer of CAX is present. R: CH_2-estradiol

Tagging and Detection

Tagging Analyte with CAX-B Followed by Detection by Direct MALDI-TOF-MS or MALDI-TOF/TOF-MS CAX-B in 50% ACN (20 mg/mL), with Et_3N (20 µL/mL) was mixed 1:1 with the sample solution (final volume 20 µL). After 2 h at 45°C, the reaction mixture was diluted with 50% ACN, mixed with CCA matrix solution in 1:10 ratio, and subjected to MALDI-TOF/TOF-MS.

Tagging Analyte with CAX-B Followed by Detection by Cap-LC/MALDI-TOF/TOF-MS Six µL of CAX-B solution (CAX-B at 1 mg/mL, and Et_3N at 10 µL/mL in 50% ACN) was added to a vial containing evaporated analyte, and after 16 h at 38°C, CapLC/MALDI-TOF/TOF-MS was done using the following LC conditions: Dionex Ultimate, with 0.3 × 150 mm, 2 µm Acclaim PepMed C18 column, mobile phase: A, H_2O with 0.1% TFA, 2% ACN; B, ACN with 0.1% TFA; gradient up to 12% B over 4 min, then up to 90% B in 40 min with collection of 3 droplets/min onto a MALDI plate, followed by manual addition of CCA matrix solution and MALDI-TOF/TOF-MS.

Detection of 160 Amol of Thymidine via CAX-Tagging /HPLC/HPLC/MALDI-TOF/TOF-MS Six µL of CAX-B solution

Figure 3. Detection of 100 (**a**) and 10 (**b**) fmol of estradiol by CAX-tagging/CapLC/MALDI-TOF/TOF-MS in a pseudo-MS3 mode. Inset: corresponding MALDI-TOF-MS spectra (**a**), (**b**) showing the molecular ion (476 Da). Note that *m/z* 100 requires a proton loss from the fragment indicated, perhaps according to the scheme shown in Figure 2

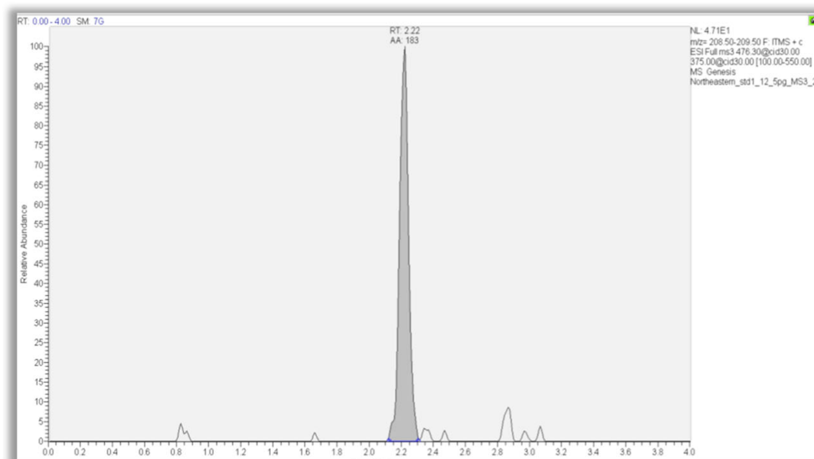

Figure 4. Detection of 63 fg (130 amol) of CAX-estradiol by HPLC/ion trap/MS in a true MS3 mode: injection of 5 μL of 12 pg/mL

(CAX-B at 1 mg/mL and Et_3N at 10 μL/mL in 50% ACN) was added to a vial containing 160 amol of evaporated thymidine, and the vial was kept for 16 h at 38°C. The solution was dried in a SPD Speed Vac (Thermo Scientific), redissolved in 5 μL of 0.1% TFA in 2% ACN, and injected into a Micro-HPLC system (Agilent 1100, Dionex PepMap 100 C18 column, 1 × 250 mm, 5 μm); flow rate: 50 μL/min; mobile phase: 12% ACN with 0.1% TFA for 4 min, then 12%–90%

Figure 5. Detection of a mixture of 12 phenols derivatized with CAX-B by MALDI-TOF-MS (50 pg of each per spot in terms of masses of the phenols and assuming a 100% reaction yield): **1**, phenol; **2**, o-cresol; **3**, 2,4-dimethylphenol; **4**, 2-chlorophenol; **5**, 2-nitrophenol; **6**, 4-nitrophenol; **7**, 4-chloro-3-methylphenol; **8**, 2,4-dichlorophenol; **9**, 2,4-dinitrophenol; **10**, 2,4,6-trichlorophenol; **11**, 2-methyl-4,6-dinitrophenol; **12**, pentachlorophenol

ACN in 40 min. The eluted sample was collected in the 8- to 17-min time window. The dried sample was re-injected into a nano-LC (Eksigent Tempo LC MALDI System (AB SCIEX), Dionex PepMap 100 C18 column, 0.075 × 150 mm, 3 μm) at 300 nL/min with an immediate gradient of 12 to 90% ACN with 0.1% TFA in 50 min with collection of 3 droplets/min onto a MALDI plate with online matrix addition (CCA matrix: 2.5 mg/mL, 0.5 mL/min syringe pump flow), followed by MALDI-TOF/TOF-MS.

Relative response of CAX-Cytosine and CAX-d_8-cytosine by MALDI-TOF-MS One μL of a solution of cytosine (8 mM) in 50% acetonitrile was combined with 10 μL from a 1 mL stock solution containing 13.7 μmol each of CAX-B and CAX-B-d_8 along with 27.4 μmol of triethylamine. After 55°C for 1 h, 1 μL of the reaction mixture was diluted with 1 mL of 50% acetonitrile, and 1 μL of that was combined with 10 μL of CCA matrix solution followed by subjecting 1 μL to MALDI-TOF-MS.

Results and Discussion

Our interest in intensified neutral loss tags, which are derivatization reagents prone to neutral loss under collision-induced dissociation conditions to yield an analyte-characteristic product ion [18], led us to the cationic mass tag reported in this paper, CAX-B. This reagent is easily prepared by reacting α,α′-dibromo-*o*-xylene with triethylamine. The derivatization of analytes having an active hydrogen with CAX-B is illustrated in Figure 1.

Initially, in preliminary studies, we prepared CAX-B along with its *meta* and *para* isomers. While all three reagents were found to label estradiol, only the estradiol derivative from the *ortho* isomer gave *m/z* 375 as a major product ion (from loss of triethylamine as a neutral). Although the details are unclear (including the contribution of steric effects), we attribute this result to anchimeric assistance. The ability of an ethereal oxygen atom to provide 1,5-anchimeric nucleophilic assistance is known [23]. However, it is conceivable that this oxygen atom in our case might also interact with a CH_2 group of a

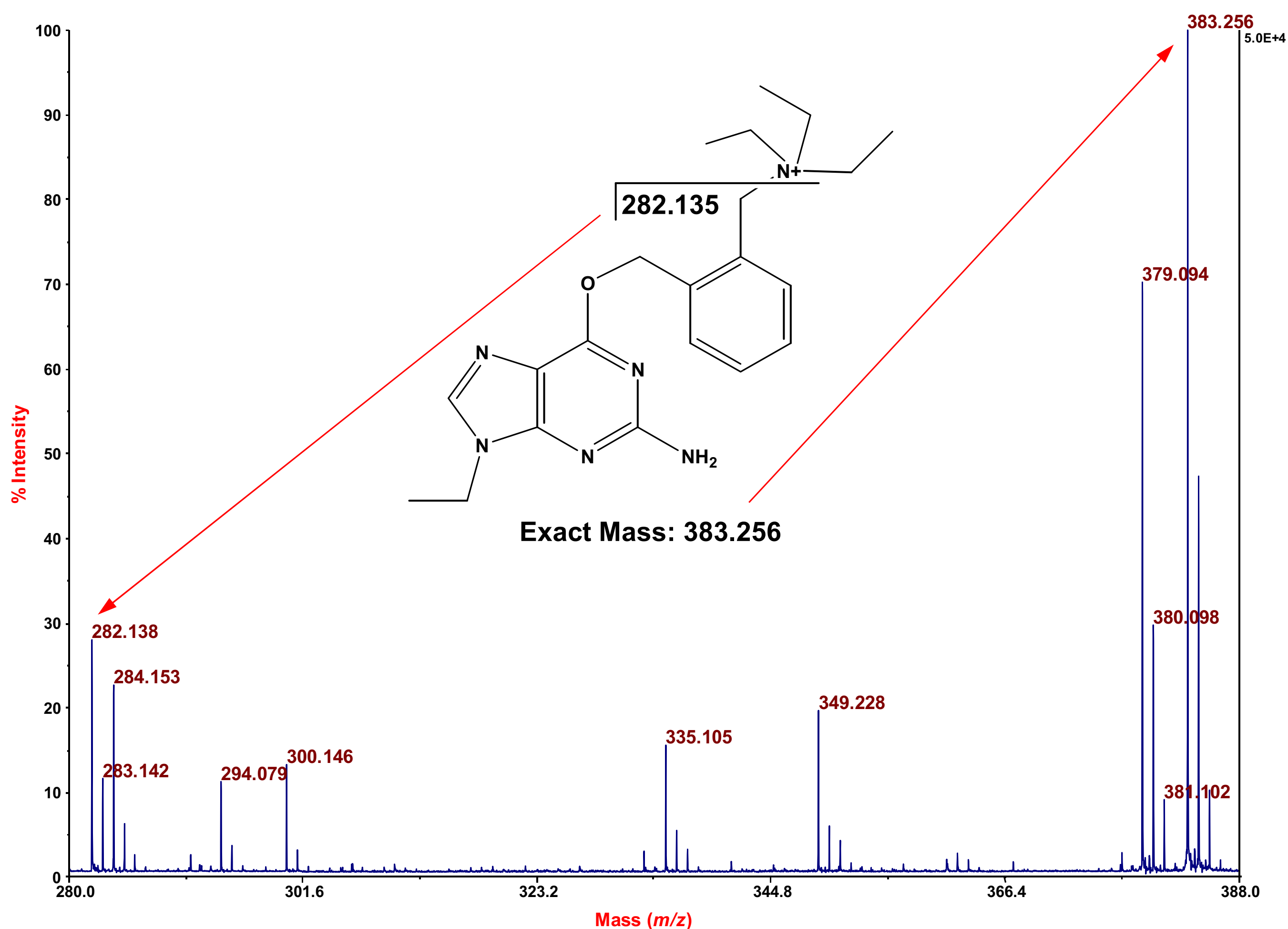

Figure 6. Detection of CAX-tagged N7-ethylguanine as both a precursor ion at 383.256 Da and as a product ion at *m/z* 282 (produced in the ion source) by MALDI-TOF-MS

triethylamine moiety of the CAX-estradiol, as suggested by an energy minimum calculation (Supplemental Figure 1). For the *meta* and *para* isomers, *m/z* 375 from derivatized estradiol was a minor product ion relative to an intense ion at *m/z* 100, which might be explained as shown in Figure 2.

When tested in a MALDI-TOF/TOF-MS under post-source decay conditions, only CAX-estradiol (referring to the *ortho* isomer of CAX) gave an intense, analyte-characteristic second product ion at *m/z* 209 (described in more detail later). This observation greatly increased our interest in CAX-B, since second product ions can boost the sensitivity and specificity of detection by tandem MS [24].

Derivatization of 100 and 10 fmol of estradiol with CAX-B, followed by CapLC/eluent spotting/MALDI-TOF/TOF-MS, gave the data shown in Figure 3, where the fragment at *m/z* 209 is defined. This was a pseudo-MS3 experiment in which a double neutral loss took place in the CID step. The exact structure of the *m/z* 209 fragment ion is unknown. Testing CAX-estradiol as a diluted standard by HPLC/ion trap/MS in a true MS3 experiment gave the data shown in Figure 4. In this experiment, 5 μL of a solution of 12.5 pg/mL (63 fg or 130 amol) was injected. Potentially, a smaller amount might be detected by employing a narrow bore column connected to nanospray.

A mixture of 12 phenols (50 ng each), varying in pKa values and steric effects attributable to different numbers and patterns of halogen and nitro substituents, was derivatized with CAX-B followed by detection by MALDI-TOF-MS. As shown in Figure 5, a fairly even response was observed (within a factor of five), taking into account multiple peaks due to the isotopes of chlorine. The peak for dinitrophenol (compound 9) is 5-fold less intense than the peak for phenol (compound 1). Because the two mononitrophenols in the mixture are isomers, the response of each is unknown.

Several nucleobases, including some modified nucleobases, were derivatized with CAX-B followed by detection as diluted standards at the amol level by MALDI-TOF/TOF-MS (Supplemental Figure 2). Observation of a precursor molecular ion at 383.256 Da, along with a product ion at *m/z* 282, is seen for CAX-N7-ethylguanine in Figure 6. When the molecular ion at

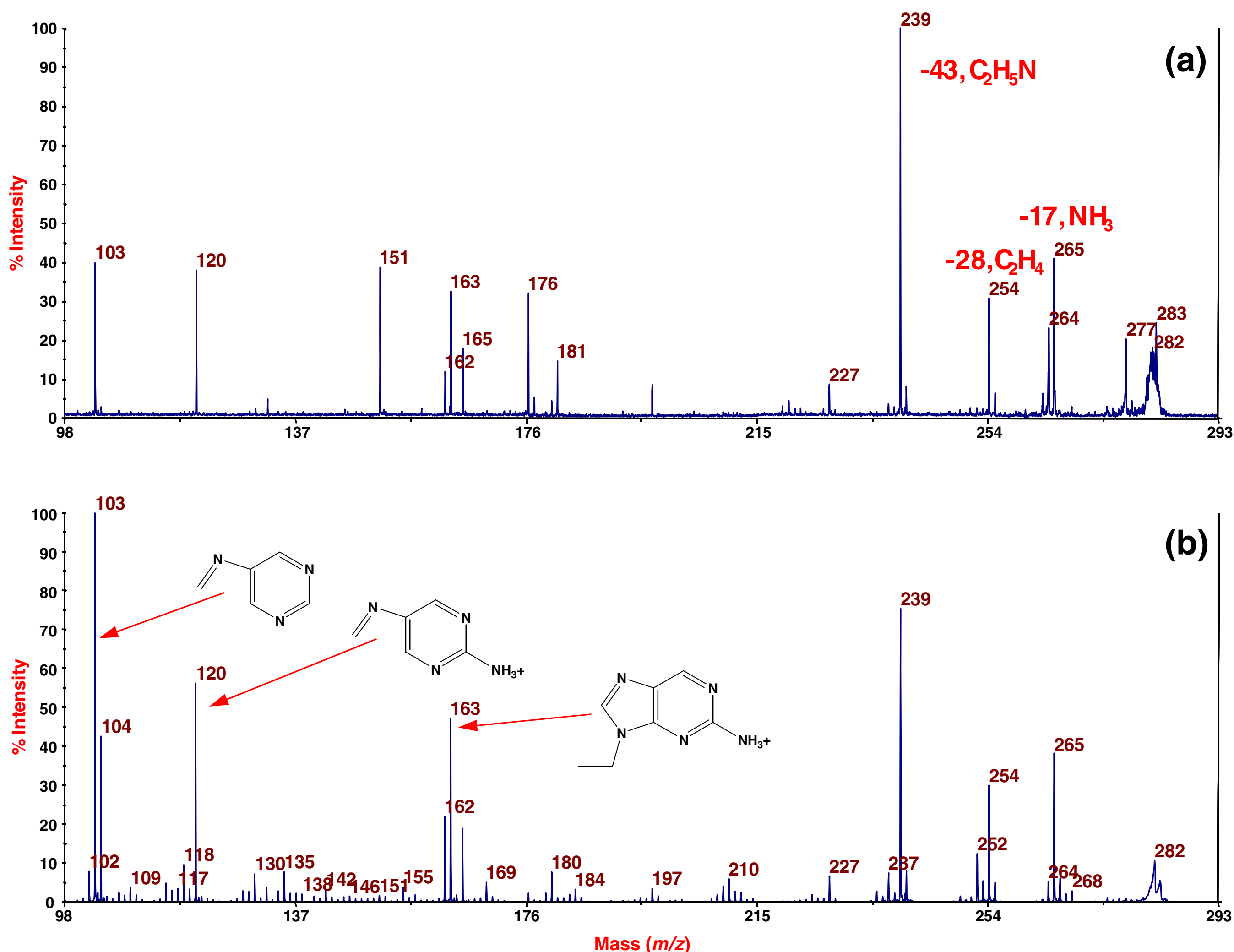

Figure 7. MALDI-TOF/TOF-MS analysis of the product ion at *m/z* 282 from CAX-tagged N7-ethylguanine: (**a**) CID off and (**b**) CID on

383 is subjected to CID, only *m*/*z* 282 is seen (data not shown). Analysis of *m*/*z* 282 (assigned in Figure 6) in a post-source decay (PSD) experiment gives the data shown in Figure 7a, whereas the analysis with CID gives richer fragmentation data than the PSD analysis as seen in Figure 7b. Now fragment ions from the nucleobase are seen.

Nucleosides possessing an active hydrogen can be favorable analytes for detection by CAX-labeling/MS3, using either pseudo MS3 (double neutral loss in CID as performed in a MALDI-TOF/TOF-MS) or in an ion trap MS. We derivatized 160 amol of thymidine with CAX-B, leading to the data shown in Figure 8. The double neutral loss (of triethylamine and deoxyribose as shown) facilitates this high sensitivity, since it leads to *m*/*z* 229. CAX-thymidine also can be detected at the amol level by LC/ion trap/MS as shown in Supplemental Figure 3.

We were able to observe molecular ions by MALDI-TOF-MS for CAX-analytes when the analyte was 2-phenylethanol (326.252 Da), 1-butanol (278.247 Da), 4-phenyl-1-butanol (354.279 Da), and deoxyribose (338.230 Da) (Supplemental Figure 4). This work was only conducted in a preliminary way, so there is no meaningful information on the best reaction conditions or the sensitivity that can be achieved. Detection and fragmentation of the CAX derivative of benzoic acid are shown in Supplemental Figure 5.

Monolabeling of bisphenol A was observed when it was reacted with CAX-B, obviously because of charge repulsion as a barrier to attachment of a second CAX moiety onto the second OH of this compound. This concept has been noted before in regard to use of pyridine as a mass tag [6]. The unusual fragmentation (in the context of this overall study) of CAX-bisphenol A is shown in Figure 9. Intense formation of a fragment ion at *m*/*z* 135, as seen, apparently is favorable since this cation is stabilized both by inductive electron donation from the two methyl groups and resonance electron donation promoted by the para OH group. Perhaps the preferred conformation of this molecule in the gas phase (unknown) also plays a role.

We prepared a deuteriated form of CAX-B by subjecting *o*-xylene-d_{10} to bromination followed by reaction with

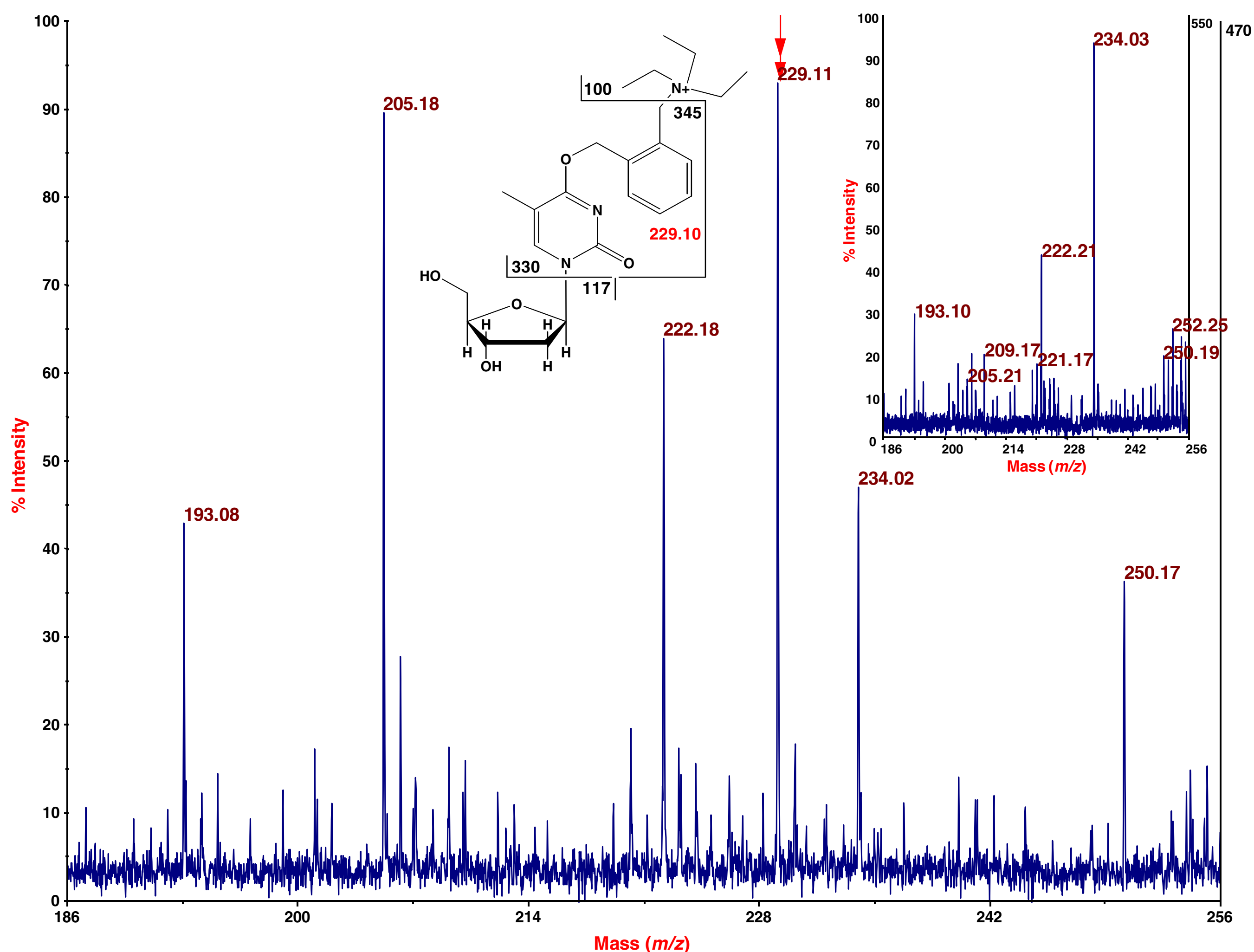

Figure 8. Detection of 160 amol of thymidine by CAX-tagging/HPLC/HPLC/MALDI-TOF/TOF-MS in a pseudo-MS3 mode. Inset: blank reaction

Figure 9. Detection of CAX-tagged bisphenol A by MALDI-TOF/TOF-MS

triethylamine. This was done to enhance specificity mainly in precursor ion detection by giving CAX-d_8 tagged analyte an unusual fragment mass for analytes of moderate mass, helping to discriminate this species from many background chemicals aside from those that are labeled as well. This is a consequence of the relatively high number of deuterium atoms in CAX-d_8. Actually, for many analytes, even the high content of H atoms of non-deuterio CAX tends to yield a somewhat unique mass fragment for a CAX-analyte of moderate mass. This is illustrated for both cases by the data shown in Figure 10, which was obtained by reacting cytosine with a 1:1 molar mixture of CAX-B and CAXC-B-d_8. As seen, the fragment masses of

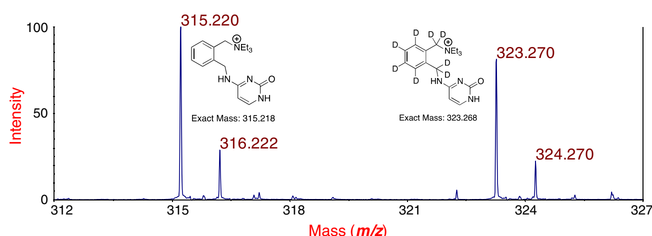

Figure 10. Detection of CAX-cytosine and CAX-d_8-cytosine by MALDI-TOF-MS after reacting cytosine with a 1:1 molar mixture of CAX-B and CAX-B-d_8

CAX-cytosine and CAX-d_8-cytosine are 0.250 and 0.301, respectively. In some cases, it will also be useful to label with a mixture of these reagents (isotopologue labeling) to enhance specificity in this way for precursor ion detection, as long as the sample is not too complex.

The advantages of CAX-B as a new cationic mass tag, whether realized or anticipated, are as follows. (1) It is easy to prepare (one step reaction from inexpensive, commercially-available starting materials). (2) It can be used to derivatize a broad range of analytes since it reacts with compounds having an active hydrogen (but this broad specificity increases the need for sample purification prior to tagging). (3) It readily gives an analyte-characteristic, first product ion in a mass spectrometer providing CID. (4) Via this latter feature, for susceptible analytes it can facilitate the formation of an analyte-characteristic second product ion to further boost sensitivity (as long as the noise falls more than the signal in going to the second product ion). (5) It tends to set up a somewhat unique fragment mass for a CAX-labeled analyte of moderate mass, to enhance specificity at the stage of precursor ion detection, and this fragment mass can be made even more unique by employing CAX-B-d_8. (6) It provides high sensitivity under both ESI and MALDI ion source conditions. (7) It tends to label an analyte of moderate size only once because of

charge repulsion. (8) Its benzylic bromine atom no doubt can be easily replaced with a variety of functional groups, leading to a family of related mass tags for more specific labeling of subgroups of analytes varying in complementary functional groups. (9) The moderate polarity of CAX can help to minimize the problem that chromatographic resolution of analytes tends to become more difficult after derivatization.

Conclusions

We conclude that CAX-B is a valuable addition to the family of cationic mass tags available for increasing the sensitivity of mass spectrometry. This is especially true when a tandem mass spectrometer is available so that one can take advantage of the ease with which a CAX-analyte forms an analyte-characteristic first product ion. In turn, a high yield of a first product ion opens up the opportunity for additional sensitivity via formation of an analyte-characteristic second product ion for some analytes. Since nucleosides possessing an active hydrogen on the nucleobase have this latter property, CAX-B may become useful for the detection of DNA adducts by mass spectrometry. CAX reagents may also become useful for metabolomics since many metabolites possess an active hydrogen (making them reactive towards CAX-B), and because additional CAX reagents are likely to emerge that can target subgroups of the metabolome in terms of functional groups.

Acknowledgments

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