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Identification of Carboxylate, Phosphate, and Phenoxide Functionalities in Deprotonated Molecules Related to Drug Metabolites via Ion–Molecule Reactions with water and Diethylhydroxyborane

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Abstract. Tandem mass spectrometry based on ion–molecule reactions has emerged as a powerful tool for structural elucidation of ionized analytes. However, most currently used reagents were designed to react with protonated analytes, making them suboptimal for acidic analytes that are preferentially detected in negative ion mode. In this work we demonstrate that the phenoxide, carboxylate, and phosphate functionalities can be identified in deprotonated molecules by use of a combination of two reagents, diethylmethoxyborane (DEMB) and water. A novel reagent introduction setup that allowed DEMB and water to be separately introduced into the ion trap region of the mass spectrometer was developed to facilitate fundamental studies of this reaction. A new reagent, diethylhydroxyborane (DEHB), was generated inside the ion trap by hydrolysis of DEMB on introduction of water. Most carboxylates and phenoxides formed a DEHB adduct, followed by addition of one water molecule and subsequent ethane elimination (DEHB adduct $+H_2O - CH_3CH_3$) as the major product ion. Phenoxides with a hydroxy group adjacent to the deprotonation site and phosphates formed a DEHB adduct, followed by ethane elimination (DEHB adduct $- CH_3CH_3$). Deprotonated molecules with strong intramolecular hydrogen bonds or without the aforementioned functionalities, including sulfates, were unreactive toward DEHB/H_2O. Reaction mechanisms were explored via isotope labeling experiments and quantum chemical calculations. The mass spectrometry method allowed the differentiation of phenoxide-, carboxylate-, phosphate-, and sulfate-containing analytes. Finally, it was successfully coupled with high-performance liquid chromatography for the analysis of a mixture containing hymecromone, a biliary spasm drug, and its three possible metabolites.

Keywords: Tandem mass spectrometry, Ion-molecule reactions, Acidic analytes, Negative ions

Introduction

Mass spectrometry (MS) is a powerful analytical technique for the identification of unknown drug metabolites within complex biological mixtures [1]. Structural information is usually obtained via MS techniques that use multiple stages of ion isolation and collision-activated dissociation (CAD) experiments [2–4]. However, MS^n based on CAD does not always provide adequate structural information for unambiguous assignment of chemical structures [5].

Tandem MS based on ion–molecule reactions allows an additional dimension of structural information to be obtained for ionized analytes [6–19]. For instance, functional-group-selective ion–molecule reactions have been developed for the rapid identification of specific functionalities, such as hydroxy [10], epoxide [11], amido [12], hydroxylamino [13], N-oxide [14], sulfoxide [15], and sulfone [16, 17], many of which are found in active pharmaceutical ingredients and their metabolites. In many cases, this approach has allowed the differentiation of isomeric ions that are not discernable via CAD experiments, making it a powerful structural elucidation tool

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complementary to CAD [18, 19]. Ion–molecule reactions can be coupled with liquid chromatography for rapid screening of compounds containing specific functionalities in complex mixtures [20, 21]. However, most of the ion–molecule reactions developed require the target analyte to be protonated [14]. Therefore, these methods are suboptimal for analytes containing acidic functional groups such as phosphate, sulfate, and glucuronide, as they are easier to ionize in negative ion mode [22]. Yet, only a few cases have been examined wherein deprotonated analytes were allowed to react with neutral reagents [19, 21, 23–25].

Diethylmethoxyborane (DEMB) is known to react with deprotonated molecules, such as phenoxides and phosphates, inside mass spectrometers [21, 24]. Deprotonated phosphocarbohydrates can be differentiated from sulfocarbohydrates as only the former compounds selectively form DEMB adduct $- CH_3OH$ on reaction with DEMB. However, DEMB cannot be used to identify carboxylates as these compounds do not produce observable products on reaction with DEMB [21]. In this work we present a novel reagent introduction system that allowed the independent introduction of two reagents, DEMB and water. The presence of water resulted in hydrolysis of DEMB to diethylhydroxyborane (DEHB), which reacts with deprotonated analytes differently from DEMB. This system can be used to differentiate multiple commonly observed acidic functionalities in deprotonated analytes, such as phenoxide, carboxylate, phosphate, and sulfate.

Method

Chemicals

DEMB (97%), benzoic acid (99.5%), 3,5-dimethoxybenzoic acid (97%), 3,4,5-trimethoxybenzoic acid (99%), phenylacetic acid (99%), *trans*-cinnamic acid (99%), octanoic acid (98%), heptanoic acid (97%), levulinic acid (98%), D-serine (98%), 2-hydroxybenzoic acid (99%), 3-hydroxybenzoic acid (99%), 2-hydroxyphenacetic acid (97%), 3-hydroxyphenacetic acid (99%), 4-methylumbelliferyl β-D-glucuronide hydrate (98%), phthalic acid (99.5%), isophthalic acid (99%), terephthalic acid (98%), phenol (99%), 4-ethoxyphenol (99%), catechol (99%), resorcinol (99%), hydroquinone (99%), 2-hydroxybenzyl alcohol (99%), 3-hydroxybenzyl alcohol (99%), phenylphosphonic acid (98%), 2-aminoethylphosphonic acid (99%), 4-methylumbelliferone (98%), 4-methylumbelliferyl phosphate (98%), 4-methylumbelliferyl sulfate potassium salt (99%), *p*-toluenesulfonic acid monohydrate (98.5%), benzenesulfonic acid (98%), morphine 6-β-D-glucuronide solution (1.0 mg/mL in methanol–water, 2:8), *p*-acetamidophenyl β-D-glucuronide sodium salt, 4-nitrophenyl β-D-glucuronide (98%), phenolphthalein β-D-glucuronide, and water-^{18}O (97 atom % ^{18}O) were purchased from Sigma-Aldrich (St Louis, MO, USA) and used as received. Water [liquid chromatography (LC)–MS grade] was purchased from ProteoChem (Hurricane, UT, USA) and used as received. Deuterium oxide (99.5%) was purchased from Cambridge Isotope Laboratories (Tewksbury, MA, USA) and used

as received. For the 4-methylumbelliferone (hymecromone) metabolite mixture, 4-methylumbelliferone, 4-methylumbelliferyl sulfate potassium salt, 4-methylumbelliferyl phosphate, and 4-methylumbelliferyl β-D-glucuronide hydrate were dissolved in 50:50 v/v methanol–water to achieve a final concentration of 0.1 mM for each compound.

Mass Spectrometry

All experiments were performed with a Thermo Scientific (Waltham, MA, USA) linear quadrupole ion trap mass spectrometer equipped with an electrospray ionization (ESI) source. Ion generation and detection was performed in negative ion mode. Analytes were dissolved in 50:50 v/v methanol–water to a final concentration of 0.5 mM. For phenol-containing analytes, 5 µL of 1 mM NaOH water solution was added to 2 mL of sample solution to facilitate the formation of the phenoxide ions on ionization. Analyte solutions were directly injected into the ESI source at a flow rate of 20 µL/min. ESI parameters were set as follows: spray voltage 3 kV, sheath gas (N_2) flow 10 (arbitrary units), and auxiliary gas (N_2) flow 5 (arbitrary units).

Ion–Molecule Reactions

Ion–molecule reactions were studied with use of a combination of a custom-built external reagent mixing manifold [8, 26] and a custom-built pulsed valve system [27]. DEMB was injected into the external reagent mixing manifold via a syringe drive at a flow rate of 3 µL/min and diluted with helium at a flow rate of 250 mL/min. The manifold was heated to 70 °C for efficient evaporation of DEMB into helium. The DEMB–helium mixture then entered a variable leak valve that allowed part of the mixture gas to enter the ion trap while the excess was directed to waste. The variable leak valve was set to maintain the pressure within the trap region of the instrument at 0.5×10^{-5} Torr (measured via an ion gauge). For introduction of H_2O into the trap region of the mass spectrometer, 5 µL of H_2O was injected via a syringe through a rubber septum into a stainless steel channel. The stainless steel channel was heated to 90 °C to promote water evaporation. A pulsed valve connected to the stainless steel channel was triggered manually via a waveform generator to open for 500 µs to allow H_2O to enter the linear quadrupole trap region. Analyte ions were isolated and allowed to react with the neutral reagents for 200 ms before being ejected for detection.
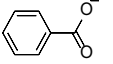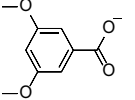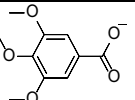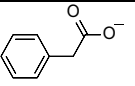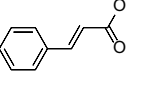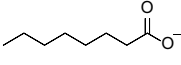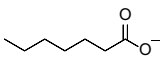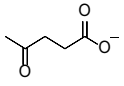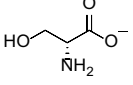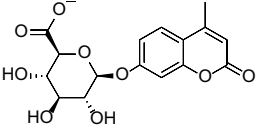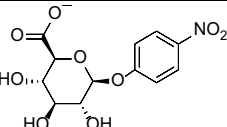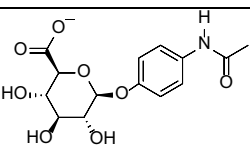
High-Performance Liquid Chromatography

High-performance LC (HPLC) experiments were performed with a Surveyor Plus HPLC system consisting of a quaternary pump, an autosampler, and a Zorbax SB-C18 column. A non-linear gradient of water with 5 mmol ammonium acetate (solvent A) and methanol with 5 mmol ammonium acetate (solvent B) was used: 0.00 min, 100% solvent A; 10.00 min, 70% solvent A and 30% solvent B; 20.00 min, 60% solvent A and 40% solvent B; 25.00 min, 30% solvent A and 70% solvent B;

30.00min, 30% solvent A and 70% solvent B; 31.00 min, 100% solvent A; 40.00 min, 100% solvent A. The flow rate of the mobile phase was kept at 500 µL/min. HPLC eluate then entered an ESI source operating in negative ion mode with the following conditions: spray voltage 3.25 kV; sheath gas (N_2) flow 50 (arbitrary units), and auxiliary gas (N_2) flow 20 (arbitrary units).

Table 1. Product ions detected after 200-ms reaction of analyte ions containing a carboxylate functionality with diethylhydroxyborane (*DEHB*) and water

Analyte ion (m/z of [M−H]⁻)	Ion structure	Products formed upon reactions with DEHB/H₂Oᵃ (m/z)
benzoic acid (121)		121 + DEHB (207) 121 + DEHB + H_2O − CH_3CH_3 (195)
3,5-dimethoxybenzoic acid (181)		181 + DEHB (267) 181 + DEHB + H_2O − CH_3CH_3 (255)
3,4,5-trimethoxybenzoic acid (211)		211 + DEHB (297) 211 + DEHB + H_2O − CH_3CH_3 (285)
phenylacetic acid (135)		135 + DEHB (221) 135 + DEHB + H_2O − CH_3CH_3 (209)
cinnamic acid (147)		147 + DEHB (233) 147 + DEHB + H_2O − CH_3CH_3 (221)
octanoic acid (143)		143 + DEHB (229) 143 + DEHB + H_2O − CH_3CH_3 (217)
heptanoic acid (129)		129 + DEHB (215) 129 + DEHB + H_2O − CH_3CH_3 (203)
levulinic acid (115)		115 + DEHB (201) 115 + DEHB + H_2O − CH_3CH_3 (189)
D-serine (104)		104 + DEHB (190) 104 + DEHB + H_2O − CH_3CH_3 (178)
4-methylumbelliferyl β-D-glucuronide (351)		351 + DEHB (437) 351 + DEHB + H_2O − CH_3CH_3 (425)
4-nitrophenyl β-D-glucuronide (314)		314 + DEHB (400) 314 + DEHB + H_2O − CH_3CH_3 (388)
p-acetamidophenyl β-D-glucuronide (326)		326 + DEHB (412) 326 + DEHB + H_2O − CH_3CH_3 (400)

ᵃData obtained for major product ions are colored *red*.

Computational Details

All density functional theory (DFT) calculations were performed with the Gaussian 09 software package [28]. Geometry optimizations were performed with the hybrid functional M06-2X with the 6-31+G(d,p) basis set. This level of theory has been successfully used in our previous studies [21]. Vibrational frequency calculations for the optimized geometries were performed at the same level of theory to obtain enthalpy values as well as to confirm that none of the minima had negative frequencies and all transition states had one negative frequency. Intrinsic reaction coordinate analyses were performed for

Table 2. Product ions detected after 200-ms reactions of analyte ions containing a phenoxide, phosphate, or sulfate functionality with diethylhydroxyborane (*DEHB*) and water

Analyte ion (m/z of [M−H]⁻)	Ion structure	Products formed upon reactions with DEHB/H₂Oᵃ (m/z)
phenol (91)		$91 + DEHB$ (177) $91 + DEHB + H_2O - CH_3CH_3$ (165)
4-ethoxyphenol (137)		$137 + DEHB$ (223) $137 + DEHB + H_2O - CH_3CH_3$ (211)
catechol (109)		$109 + DEHB - CH_3CH_3$ (165)
resorcinol (109)		$109 + DEHB$ (195) $109 + DEHB + H_2O - CH_3CH_3$ (183)
hydroquinone (109)		$109 + DEHB$ (195) $109 + DEHB + H_2O - CH_3CH_3$ (183)
2-hydroxybenzyl alcohol (123)		$123 + DEHB - CH_3CH_3$ (179)
3-hydroxybenzyl alcohol (123)		$123 + DEHB$ (209) $123 + DEHB + H_2O - CH_3CH_3$ (197)
phenylphosphonic acid (157)		$157 + DEHB - CH_3CH_3$ (213)
2-aminoethylphosphonic acid (124)		$124 + DEHB - CH_3CH_3$ (180)
4-methylumbelliferyl phosphate (255)		$255 + DEHB - CH_3CH_3$ (311)
4-methylumbelliferyl sulfate (255)		none
p-toluenesulfonic acid (171)		none
benzenesulfonic acid (157)		none

ᵃThe text DEHB adduct−CH₃CH₃ is colored *red*.

all transition states to confirm that they connect to the correct reactant and product.

Results and Discussion

Analyte ions containing a phenoxide, carboxylate, phosphate, or sulfate functionality were generated by negative ion mode ESI via deprotonation and isolated inside a linear quadrupole ion trap. Their reactions with DEMB as well as DEHB/H_2O were studied. The observed reactions are summarized in Eqs. 1, 2, 3, 4, 5, and 6 (the products formed on reaction with DEHB/H_2O are highlighted in red):

phenoxides + DEMB → DEMB adduct (1)

phenoxides with adjacent hydroxy groups and phosphates + DEMB → DEMB adduct – CH₃OH (2)

carboxylates and sulfates + DEMB → no products (3)

carboxylates and phenoxides + DEMB/H_2O → DEHB adduct + H_2O – CH₃CH₃ (4)

phenoxides with adjacent hydroxy groups and phosphates → DEHB adduct – CH₃CH₃ (5)

sulfates + DEMB/H_2O → no products (6)
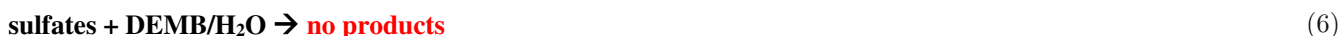
DEMB was introduced into the ion trap continuously via a reagent mixing manifold and was maintained at a constant concentration inside the ion trap, whereas H_2O was introduced into the instrument by the triggering of a pulsed valve when desirable.

Table 3. Product ions detected after 200-ms reaction of analyte ions containing both a carboxylate functionality and another acidic functionality with diethylhydroxyborane (*DEHB*) and water

Analyte ion (m/z of [M−H]⁻)	Ion structure	Products formed upon reactions with DEHB/H_2Oᵃ (m/z)
phthalic acid (165)		none
isophthalic acid (165)		165 + DEHB (251) 165 + DEHB + H_2O − CH₃CH₃ (239)
terephthalic acid (165)		165 + DEHB (251) 165 + DEHB + H_2O − CH₃CH₃ (239)
2-hydroxybenzoic acid (137)		None
3-hydroxybenzoic acid (137)		137 + DEHB (223) 137 + DEHB + H_2O − CH₃CH₃ (211)
2-hydroxyphenylacetic acid (151)		none
3-hydroxyphenylacetic acid (151)		151 + DEHB (237) 151 + DEHB + H_2O − CH₃CH₃ (225)

ᵃ Data obtained for major product ions are colored *red*.

Without the presence of H_2O, DEMB reacted with the deprotonated analytes in a manner expected from the literature [21, 24]. In short, phenoxides formed stable DEMB adducts, whereas phenoxides with a hydroxyl group near the deprotonation site and phosphates formed DEMB adduct $-$ CH_3OH (Table S1). Carboxylates (including benzoates) and sulfates did not form detectable product ions. When H_2O was introduced into the ion trap in pulses, DEMB was hydrolyzed to DEHB. Most of ions mentioned yielded detectable product ions on reaction with DEMB (Tables 1, 2, and 3). Furthermore, phenoxides, carboxylates, and phosphates yielded different product ions, whereas sulfates were unreactive.

To illustrate the observed reactivity, catechol will be used as an example. Figure 1 shows mass spectra collected after reactions of deprotonated catechol with the reagents. Deprotonated catechol (m/z 109) reacted with DEMB by addition accompanied by elimination of CH_3OH (m/z 177), as reported earlier [21]. The abundance of the DEMB adduct $-$ CH_3OH product ion did not change over time, as expected, because DEMB

concentration was held constant. Triggering of the pulsed valve caused H_2O to enter the ion trap, resulting in an increase in the abundance of a new product ion (m/z 165) containing boron (based on boron isotope distribution), which was not observed before introduction of H_2O. This ion was later identified as DEHB adduct $-$ CH_3CH_3. At the same time, a corresponding decrease in the abundances of the analyte ion (deprotonated catechol of m/z 109) and the DEMB $-$ CH_3OH product ion (m/z 177) was observed. Therefore, it can be concluded that the formation of the product ion of m/z 165 was reliant on the introduction of H_2O. The mechanism of this reaction is discussed later.

Formation of DEHB Adduct + H_2O $-$ CH_3CH_3 for Carboxylates and Phenoxides

Most analyte ions containing an aliphatic or aromatic carboxylate functionality did not form observable product ions with DEMB (Table S1), in agreement with the findings of an earlier
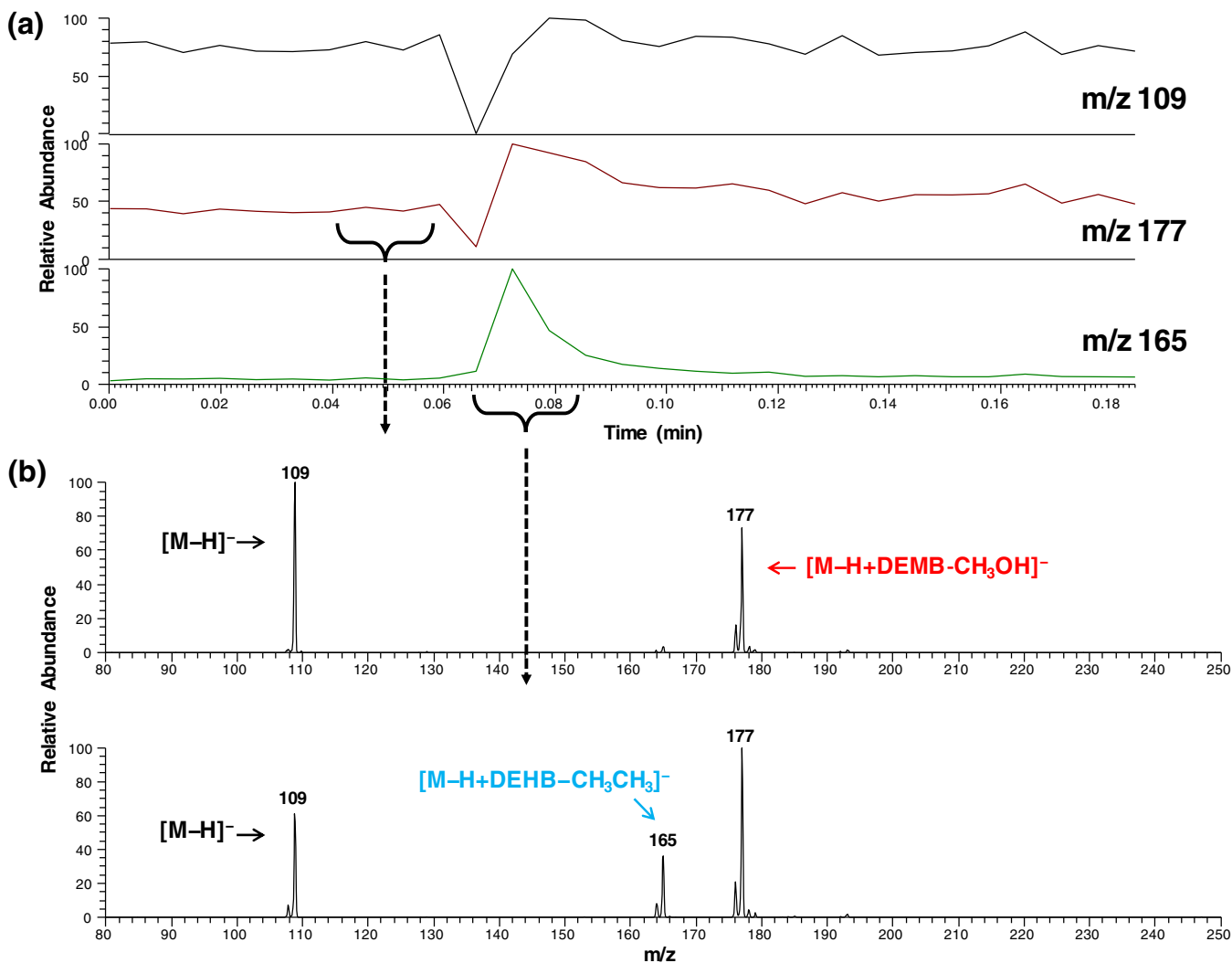

Figure 1. **a** Signals for the reactant ion (m/z 109), diethylmethoxyborane (*DEMB*) product ion (m/z 177), and diethylhydroxyborane (*DEHB*)/H_2O product ion (m/z 165) over time for reactions of deprotonated catechol (m/z 109) with DEMB and DEMB/H_2O (*a*), and averaged mass spectra measured before (*top*) and after (*bottom*) pulsed introduction of H_2O (*b*)

study [21]. However, they did undergo reactions after introduction of water, producing two types of product ions (Tables 1 and 3). To elucidate the structures of the product ions and gain a better understanding of their formation mechanisms, deprotonated benzoic acid (m/z 121) was allowed to react with DEMB/H_2O, DEMB/D_2O, and DEMB/$H_2{}^{18}O$ (Figure 2a). For unlabeled H_2O, product ions of m/z 195 and 207 (with a mass difference of 12 u) were observed, whereas product ions of m/z 197 and 208 (with a mass difference of 11 u) were observed for D_2O, and ions of m/z 199 and 209 (with a mass difference of 10 u) were observed for $H_2{}^{18}O$. These results suggest that the larger product ions (m/z 207, 208, and 209) contain one hydrogen atom and an oxygen atom that originate from water, and hence that one hydrogen atom that originates from water has been eliminated.

These findings are in agreement either with reaction of DEMB with water to form DEHB via elimination of CH_3OH

(containing one hydrogen atom originating from water), which then forms a stable adduct with the benzoate ion analyte, or with reaction of the benzoate ion with DEMB to form a stable adduct that then reacts with water to eliminate a methanol molecule (containing one hydrogen atom originating from water). As benzoic acid is unreactive toward DEMB, the former possibility appears likelier. Furthermore, the product formed on reaction of phenoxides with DEMB, a DEMB adduct, was found to be unreactive toward water (Fig. S8). Hence, the observed reactions actually involve the DEHB and H_2O reagent system rather than DEMB and H_2O. In the following discussion, terms such as "product ions formed on reaction with DEHB/H_2O" refer to ions that were observed only after introduction of H_2O.

Further insight into the reaction was obtained by examination of the m/z values of the smaller product ions formed on reaction of benzoate with DEHB/H_2O, DEHB/D_2O, and
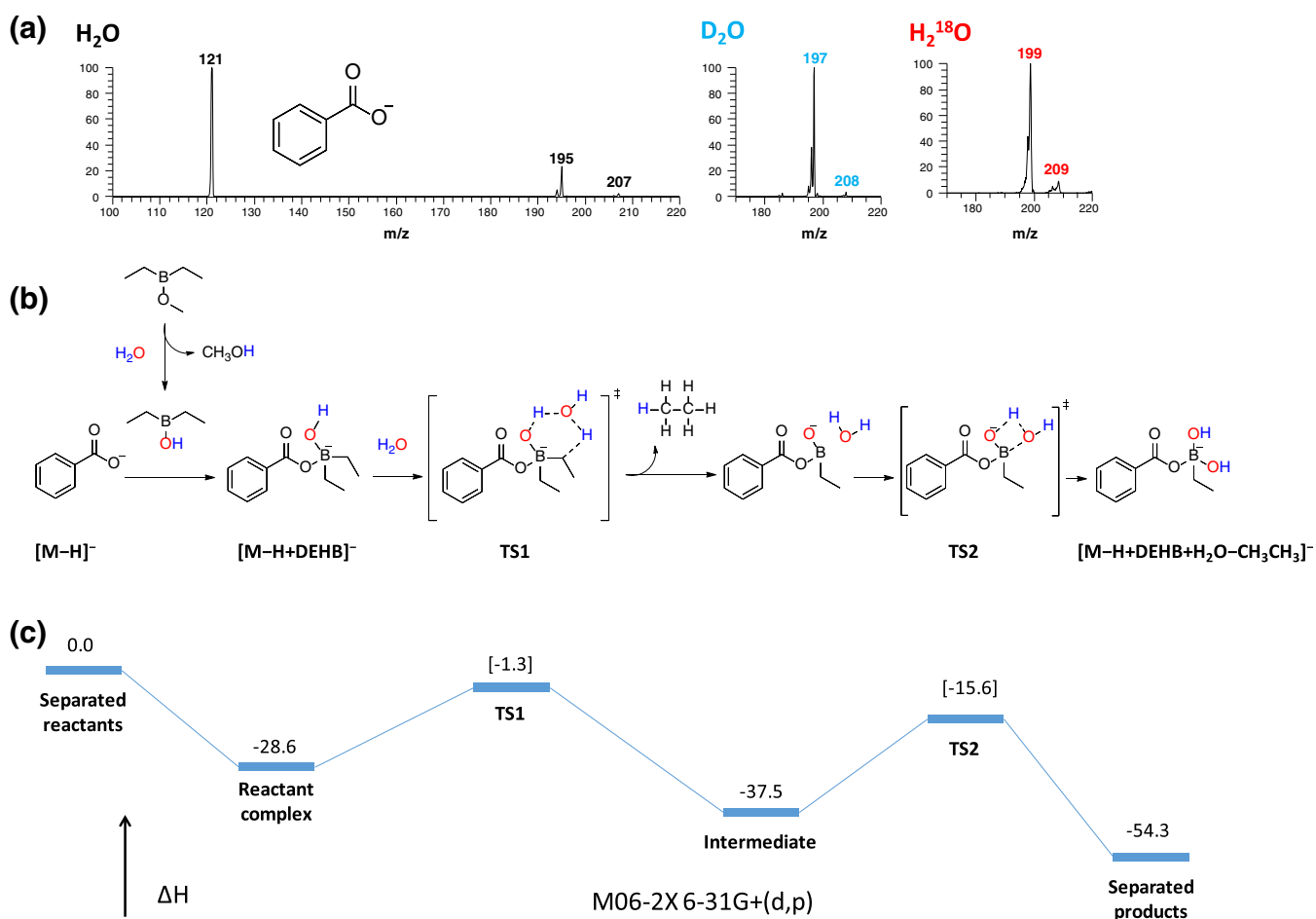

Figure 2. **a** Mass spectrum measured after 200-ms reactions of deprotonated benzoic acid (m/z 121) with DEHB/H_2O (*left*), DEHB/D_2O (*middle*), and DEHB/$H_2{}^{18}O$ (*right*). Two product ions were observed: an ion of m/z 207/208/209 corresponding to a stable adduct of deprotonated benzoic acid and DEHB, and an ion of m/z 195/197/199 corresponding to a DEHB adduct + H_2O − CH_3CH_3. **b** Proposed mechanism for the formation of DEHB adduct + H_2O − CH_3CH_3 product ion (m/z 195) on reaction of deprotonated benzoic acid with DEHB/H_2O. Hydrogen and oxygen atoms that originate from the H_2O reagent are colored *blue* and *red* respectively. **c** Calculated potential energy surface (enthalpy in kilocalories per mole) for the mechanism shown in **b** (M06-2X/6-31G+(d,p) level of theory). The separated reactants correspond to DEHB, benzoate anion, and H_2O. The three-dimensional structures of transition state 1 (*TS1*) and transition state 2 (*TS2*) are provided in Fig. S7.

DEHB/$H_2^{18}O$ (Figure 2a); that is, m/z 195, 197, and 199 (Figure 2a). The observed m/z values suggest that the smaller product ions contain two hydrogen atoms and two oxygen atoms that originate from the H_2O reactant.

In light of these findings, a pathway involving two water molecules is proposed to explain the formation of the two product ions for carboxylates (Figure 2b). On the basis of this pathway, the reaction is initiated by the hydrolysis of DEMB to DEHB. This is followed by addition of DEHB to the analyte ion to generate a DEHB adduct ion (a stable adduct was observed for all carboxylates; Table 1), the larger of the two

observed product ions. Reaction of this adduct ion ([M − H + DEHB]⁻) with H_2O leads to the elimination of an ethane molecule, yielding the smaller product ion, DEHB adduct + H_2O − CH_3CH. DFT calculations were conducted to evaluate the proposed mechanism (Figure 2c). The highest barrier found for the formation of DEHB adduct + H_2O − CH_3CH_3 for benzoate ion was −0.3 kcal/mol, suggesting that the aforementioned mechanism is energetically feasible.

The mechanism requires the presence of a large number of water molecules in the trap after the pulsed valves have been opened. Evidence in support of this expectation is as follows.
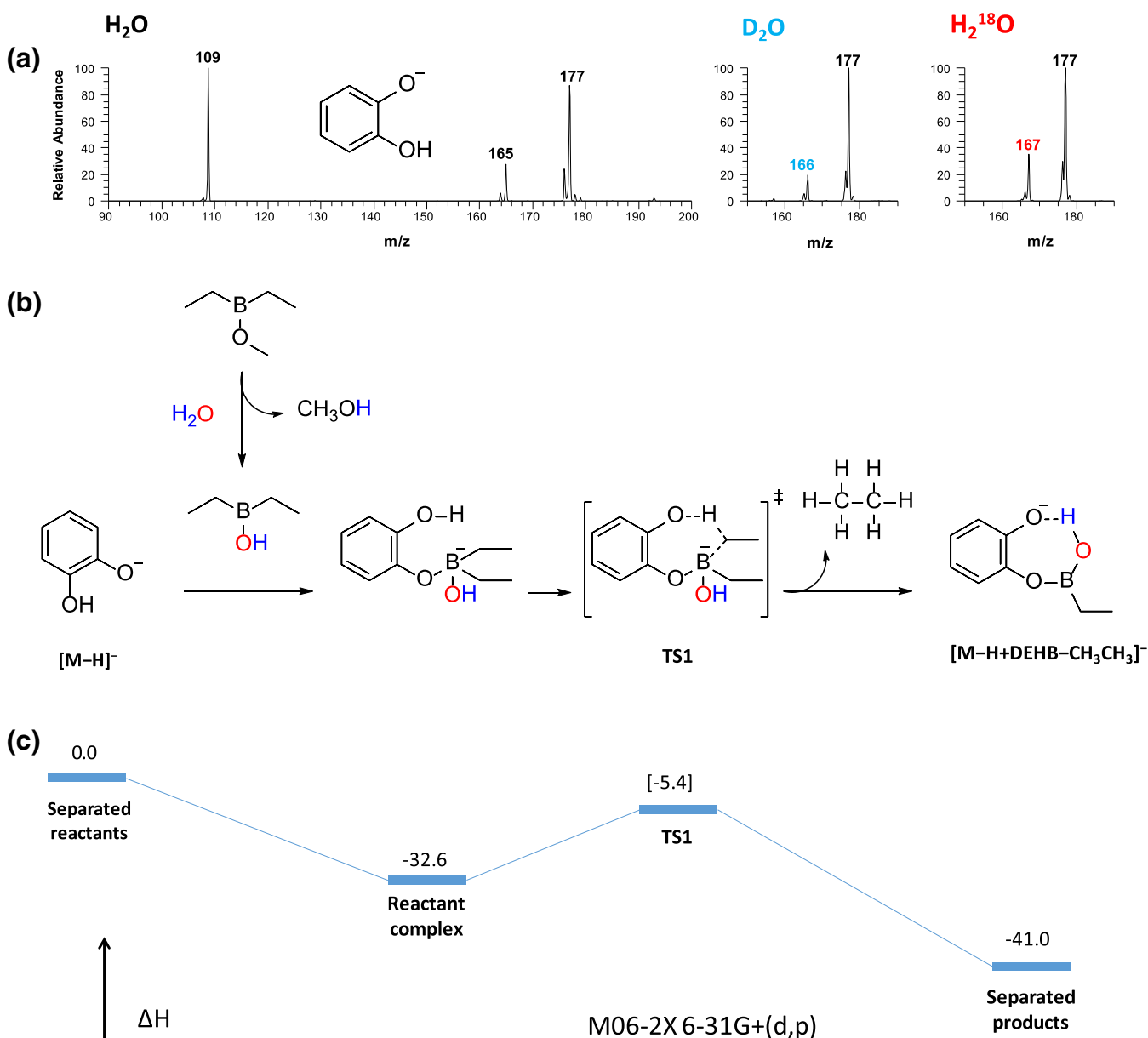

Figure 3. **a** Mass spectrum measured after 200-ms reactions of deprotonated catechol (m/z 109) with DEHB/H_2O (*left*), DEHB/D_2O (*middle*), and DEHB/$H_2^{18}O$ (*right*). **b** Proposed mechanism for the formation of DEHB adduct – CH_3CH_3 product ion on reactions between deprotonated catechol and DEHB/H_2O. Hydrogen and oxygen atoms that originate from the H_2O reactant are colored *blue* and *red* respectively. **c** Calculated potential energy surface (enthalpy in kilocalories per mole) for the mechanism shown in **b** (M06-2X/6-31G+(d,p) level of theory)

When water is introduced into the ion trap in pulses, all reactant ions disappear from the trap (e.g., see Figure 2), indicating that all DEMB molecules in the trap at that point have been converted into DEHB molecules. Further, also the product ions requiring the presence of DEMB have almost entirely disappeared (see Figure 2), providing further support for the absence of DEMB molecules in the trap immediately after water has been introduced in pulses. A third piece of evidence comes from our calculations regarding hydrolysis of DEMB to DEHB. This hydrolysis is calculated to be feasible only when two water molecules participate in the transition state (Fig. S3), thus requiring a very large amount of water in the trap. The hydrolysis of DEMB is expected to be faster than hydrolysis of DEHB. If the two reactions had similar kinetics, one would expect some ethyldihydroxyborane to be formed. The hydroxy group may stabilize DEHB with respect to hydrolysis.

Most analyte ions containing the phenoxide functionality also formed a stable DEHB adduct and a DEHB adduct $+ H_2O - CH_3CH_3$ product ion on reaction with DEHB/H_2O (Table 2, Fig. S1). The results obtained with isotopically labeled water (D_2O and $H_2{}^{18}O$) were analogous to those reported for carboxylates (Fig. S1). Therefore, phenoxides and carboxylates are assumed to form the DEHB adduct and DEHB adduct $+ H_2O - CH_3CH_3$ product ions via a similar mechanism (Fig. S1). It is worth mentioning that the DEHB/H_2O reagent system can still be used to differentiate phenoxides and carboxylates as only phenoxides form the characteristic DEMB adduct before introduction of H_2O as the result of reaction with DEMB [21] (Fig. S2, Table S1).

Formation of DEHB Adduct − CH₃CH₃ for Phenoxides with an Adjacent Hydroxy Group and Phosphates

A new product ion, DEHB adduct $- CH_3CH_3$, was observed for deprotonated catechol and deprotonated 2-hydroxybenzyl alcohol on their reactions with DEHB/H_2O (Table 2). Analogous product ion was not observed for deprotonated resorcinol, hydroquinone, or 3-hydroxybenzyl alcohol, which all have a hydroxy group that is located further away from the phenoxide group. Therefore, a hydroxy group adjacent to the phenoxide group must participate in the formation of this new product ion.

Experiments wherein D_2O and $H_2{}^{18}O$ were used instead of H_2O revealed that the DEHB adduct $- CH_3CH_3$ product ion contains one hydrogen atom and one oxygen atom from the H_2O reagent (Figure 3a). In addition, the product ion dissociates back to the analyte ion on CAD (likely via loss of O=B–CH_2CH_3), suggesting that the linkage formed between the neutral reagent and the analyte ion is relatively weak (Fig. S4). A mechanism consistent with these observations was proposed (Figure 3b) and evaluated via DFT calculations (Figure 3c).

Formation of the DEHB adduct−CH_3CH_3 product ions was also observed for phosphates. Also these ions contain a hydroxy group close to the deprotonation site as the deprotonated phosphate group contains a hydroxy group. A reaction mechanism was proposed on the basis of isotope labeling experiments that confirmed that the DEHB adduct $- CH_3CH_3$ observed for deprotonated phenylphosphonic acid contains one
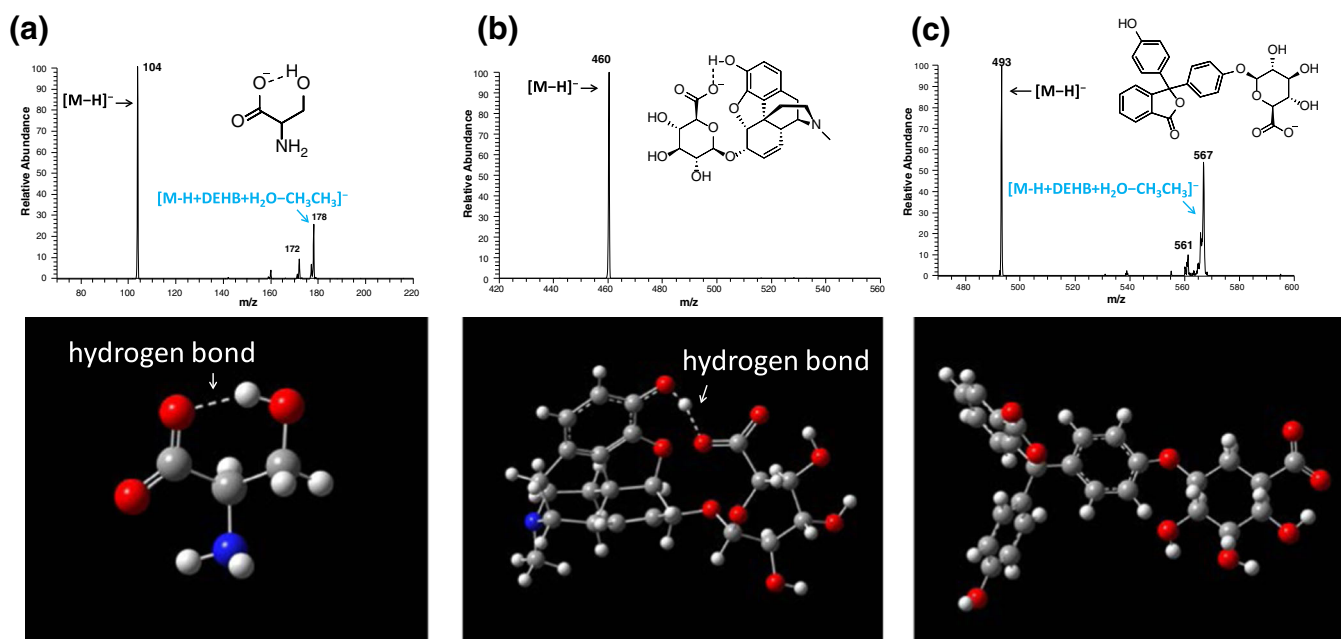

Figure 4. *Top*: Mass spectra measured after reactions between (**a**) deprotonated D-serine with DEHB/H_2O for 200 ms, **b** deprotonated morphine 6-β-D-glucuronide with DEHB/H_2O for 1000 ms, and **c** deprotonated phenolphthalein β-D-glucuronide with DEHB/H_2O for 200 ms. *Bottom:* Optimized gas-phase conformations for **a** deprotonated D-serine (hydrogen bond 1.70 Å), **b** deprotonated morphine 6-β-D-glucuronide (hydrogen bond 1.61 Å), and **c** deprotonated phenolphthalein β-D-glucuronide calculated with density functional theory at the M06-2X/6-31+(d,p) level

hydrogen atom and one oxygen atom originating from the H_2O reactant (Fig. S5). Sulfates do not contain a hydroxy group near the deprotonation site and hence do not yield these product ions, and sulfates were found to be unreactive toward DEHB/H_2O (Table 2).

False Negative Results Caused by Intramolecular Hydrogen Bonds

Not all analyte ions that contain the carboxylate or the phenoxide functionality reacted with DEHB/H_2O. Deprotonated 2-hydroxybenzoic acid, 2-hydroxyphenylacetic acid, and phthalic acid exhibited no reactivity toward DEHB/H_2O, whereas their isomers, 3-hydroxybenzoic acid, 3-

hydroxyphenylacetic acid, and isophthalic acid, formed the DEHB adduct + H_2O − CH_3CH_3 product ions as expected (Table 3). The analyte ions that showed no reactivity contain an acidic functionality (phenol or carboxylic acid) near the deprotonation site. Therefore, the intramolecular hydrogen bond formed between the acidic functionalities and the deprotonation site likely prevents reactions with DEHB/H_2O by reducing the nucleophilicity of the ions.

This hypothesis is supported by the observation that weaker intramolecular hydrogen bonds do not prevent reactions. For example, deprotonated serine forms a hydrogen bond between its carboxylate and hydroxy groups, yet DEHB adduct + H_2O − CH_3CH_3 product ions were observed on its reaction with DEHB/H_2O (Figure 4a). This is likely due to hydroxy groups
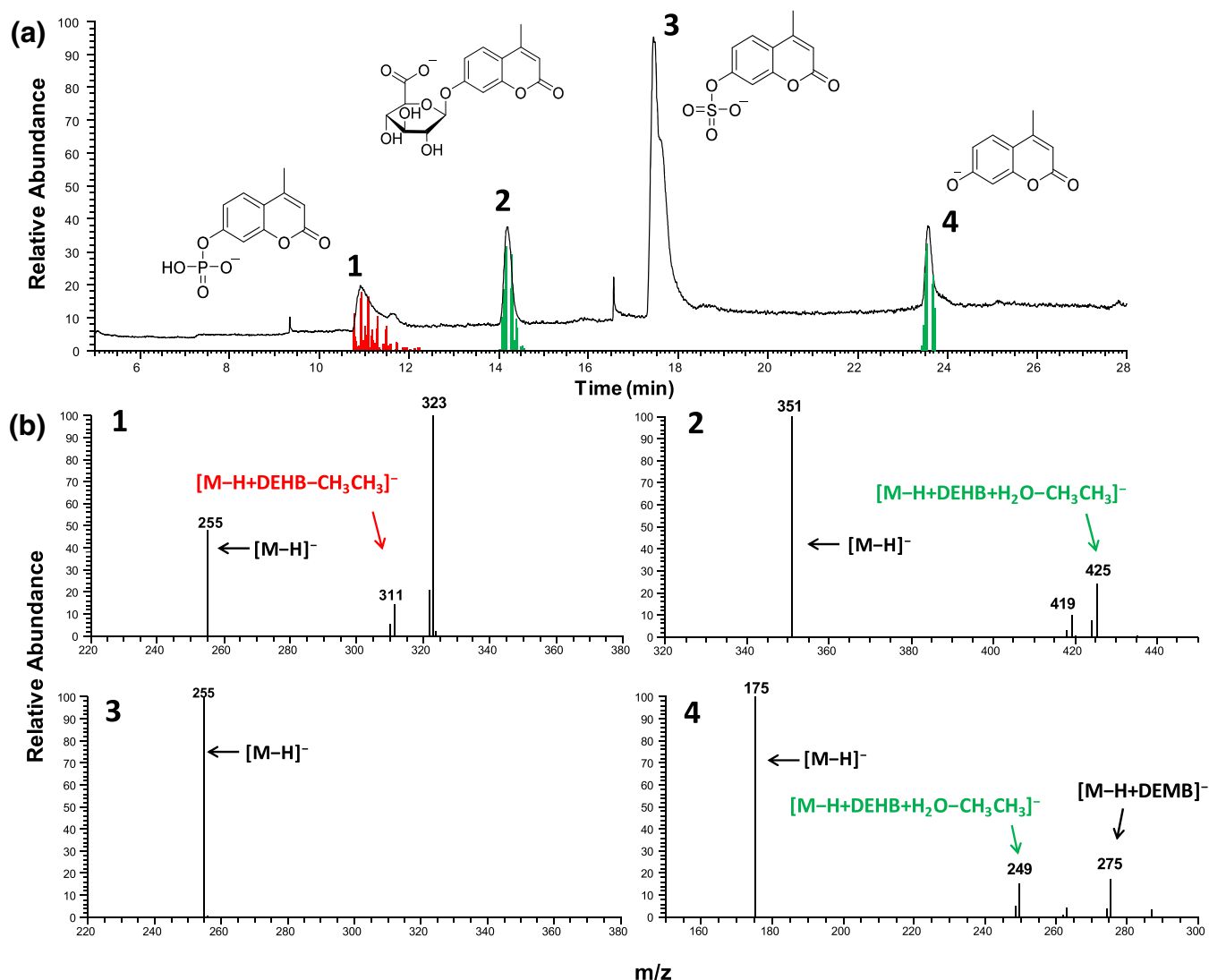

Figure 5. **a** High-performance liquid chromatogram measured for an artificial mixture of 4-methylumbelliferyl phosphate (*1*), 4-methylumbelliferyl β-D-glucuronide (*2*), 4-methylumbelliferyl sulfate (*3*), and 4-methylumbelliferone (*4*). The total ion signal is plotted in *black*. The signals measured for ion–molecule reaction product ions with DEHB/H_2O are plotted in *red* (DEHB adduct – CH_3CH_3) and *green* (DEHB adduct + H_2O – CH_3CH_3). **b** Mass spectra measured after reactions between the deprotonated analytes [4-methylumbelliferyl phosphate (*1*), 4-methylumbelliferyl β-D-glucuronide (*2*), 4-methylumbelliferyl sulfate (*3*), and 4-methylumbelliferone (*4*)] with DEHB/H_2O for 200 ms

being less acidic than phenol or carboxylic acid groups, thus forming weaker hydrogen bonds [29] that are not strong enough to prevent reactions with DEHB/H_2O.

These observations indicate the possibility of obtaining false negative results in the identification of the carboxylate functionality for certain analytes. For example, deprotonated morphine 6-glucuronide did not react with DEHB/H_2O despite containing a carboxylate functionality (Figure 4b). DFT calculations revealed that the glucuronide moiety in deprotonated morphine 6-glucuronide can rotate to a position that forms a strong hydrogen bond between the carboxylate group and the phenol group at the 3-position. In contrast, deprotonated phenolphthalein β-D-glucuronide formed the DEHB adduct + H_2O − CH_3CH_3 product ion as its rigid structure prevents the formation of a hydrogen bond between its carboxylate and phenol groups (Figure 4c).

Coupling Ion–Molecule Reaction Methodology with HPLC

HPLC separation before MS experiments is necessary for the analysis of mixtures containing isobaric or isomeric analytes. The feasibility of coupling ion–molecule reaction experiments involving DEHB/H_2O with HPLC was tested with use of an artificial mixture containing 4-methylumbelliferone (hymecromone), a choleretic and antispasmodic drug [30], and its three metabolites: 4-methylumbelliferyl phosphate, 4-methylumbelliferyl sulfate, and 4-methylumbelliferyl β-D-glucuronide (Figure 5a). The mixture was separated by reversed-phase HPLC, ionized in negative ion ESI mode, and subjected to ion–molecule reactions with DEHB/H_2O for 200 ms. On elution of each analyte, H_2O was introduced into the ion trap in pulses every 6 s for the duration of the HPLC peak. A typical HPLC peak was 15–30 s wide. Hence, two to five H_2O pulses were used for each peak. Five to ten individual ion–molecule reaction experiments were performed within each pulse. Deprotonated 4-methylumbelliferyl phosphate formed the characteristic DEHB adduct − CH_3CH_3 product ion, whereas its isobar, 4-methylumbelliferyl sulfate, showed no reactivity (Figure 5b). Both deprotonated 4-methylumbelliferone (contains a phenoxide functionality) and 4-methylumbelliferyl β-D-glucuronide (contains a carboxylate functionality) formed the DEHB adduct − CH_3CH_3 product ion. However, they can be differentiated as deprotonated 4-methylumbelliferone contains a phenoxide group and hence forms the characteristic DEMB adduct on reaction with DEMB (Figure 5b).

Conclusions

A tandem MS method was developed that allowed independent introduction of two reagents, DEMB and H_2O, into an ion trap. This setup was used to examine the reactions of deprotonated analytes with DEMB as well as with DEHB/H_2O essentially simultaneously. A new neutral reagent, DEHB, was generated inside the ion trap on introduction of both DEMB and H_2O that exhibited reactivities different from those of DEMB toward anions containing a phenoxide, carboxylate, phosphate, and sulfate functionality. On reaction with DEHB/H_2O, carboxylates and phenoxides formed DEHB adduct + H_2O − CH_3CH_3 product ions, whereas phenoxides with an adjacent phenol or hydroxymethyl group and phosphates formed DEHB adduct − CH_3CH_3. Sulfates and ions that form strong intramolecular hydrogen bonds were unreactive. Coupling of the aforementioned technique with HPLC allowed the identification of the functional groups in multiple metabolites of a drug molecule, demonstrating the potential of tandem MS based on ion–molecule reactions as a powerful analytical tool for drug metabolite identification in mixtures.

Acknowledgements

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