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JMS Letters

Dear Sir,

Distinction of *N*-Substituted Histidines by Electrospray Ionization Mass Spectrometry

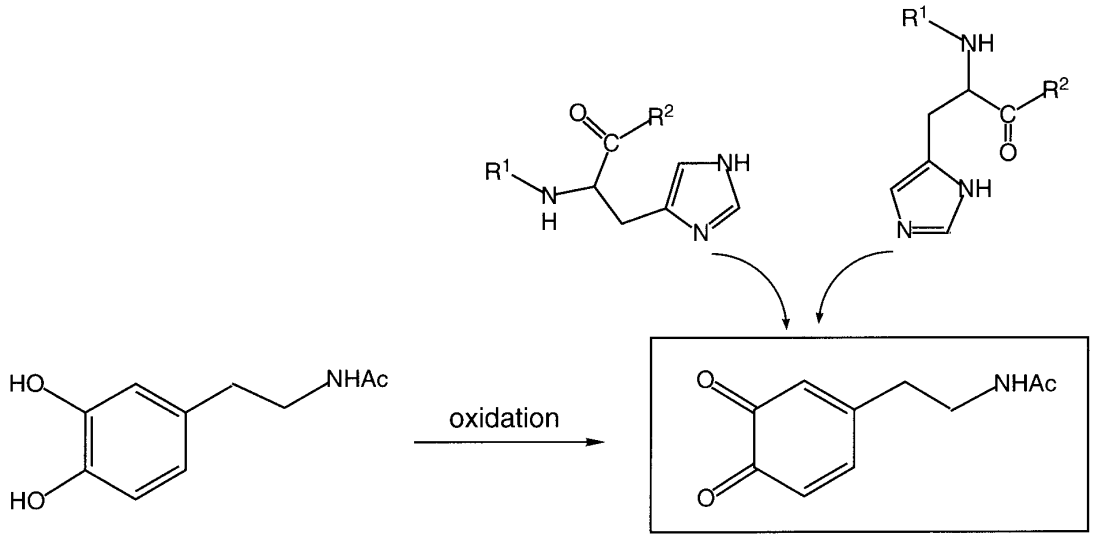
The amino acid histidine is one of the primary sites of protein conjugation with catecholamines that occurs during the process of insect cuticle sclerotization.[1,2] Oxidative conjugation of the model compounds *N*-acetylhistidine (NAcHis) with *N*-acetyldopamine (NADA) via an *o*-quinone intermediate (NADA quinone) has been studied previously and the site of NAcHis attachment at the aromatic ring of NADA has been elucidated by NMR spectroscopy.[3] Analysis of products derived from the acid hydrolysis of insect cuticle provided further evidence for the cross-links between catecholamines and histidyl residues of the cuticular proteins.[4,5] A more difficult task is to establish the site of catecholamine attachment at the histidine moiety, for which two different nucleophilic groups, N^{τ} and N^{π}, can react with *N*-acetyldopaquinone (Scheme 1). Because of the substitution pattern in the imidazole ring, conjugates formed via nucleophilic attack by the non-equivalent imidazole nitrogen atoms (N^{τ} and N^{π}) are difficult to distinguish by 2D-NMR without the assistance of molecular modeling and with small quantities of adducts obtained from the reaction mixture by reversed-phase liquid chromatography.[4] Here, we report a facile distinction of N-1 (N^{τ}) and N-3 (N^{π}) substituted histidines using electrospray ionization tandem mass spectrometry (ESI-MS/MS). A mechanistic rationale for the distinct dissociations of the gas-phase ions is also discussed.

As model compounds, we used N-1 (N^{τ}) and N-3 (N^{π}) methylated histidines **1** and **2** (purchased from Sigma and Aldrich, respectively, and used as received). The compounds were infused in methanol or aqueous methanol solutions containing 0.5% acetic acid or ammonium acetate. ESI-MS of **1** and **2** resulted in efficient protonation to yield ions $\mathbf{1H}^+$ and $\mathbf{2H}^+$, respectively, which appeared at *m/z* 170 (spectra not shown). Collisionally activated dissociations (CAD) of $\mathbf{1H}^+$ and $\mathbf{2H}^+$ were investigated in a radiofrequency-only quadrupole collision cell of a Sciex API-III triple-quadrupole tandem mass spectrometer (argon as collision gas, 26 eV laboratory collision energy) and in a quadrupole ion trap (Finnigan LCQ, helium at 10^{-3} Torr (1 Torr = 133.3 Pa) as buffer gas). The N-1 methylated isomer $\mathbf{1H}^+$ underwent dominant loss of $H_2O + CO$ to form a fragment ion at *m/z* 124. This dissociation had a sharp energy threshold; the *m/z* 124 peak was weak at low collision energies, e.g., ~1% relative to *m/z* 170 at ~0.5 eV, corresponding to 5% relative collision energy (RCE) in the ion trap instrument. However, at 10% RCE (~1 eV), CAD was almost complete and the *m/z* 124 ion was by far the predominant species in the spectrum [Fig. 1(a)]. Other primary fragments of $\mathbf{1H}^+$ were weak, e.g., *m/z* 153 (loss of ammonia), 152 (loss of water) and 126 (loss of CO_2). A secondary fragment appeared at *m/z* 109, which was due to sequential elimination of CO_2 and NH_3. To deduce the elemental composition of the *m/z* 109 ion, the [^{13}C,^{15}N]-isotopomer of $\mathbf{1H}^+$ at *m/z* 171 was selected and collisionally dissociated. The relative abundances of the *m/z* 110 and 109 ions (82.9 and 17.1%, respectively, data not shown) were close to those calculated for loss of $CO_2 + NH_3$ (83.3 and 16.7%, respectively), but differed from those calculated for loss of $CO + H_2O + CH_3$ (75.0 and 25.0%, respectively). By comparison, the measured relative abundances of the *m/z* 125 and 124 ions (87.0 and 13.0%, respectively) agreed well with the calculated values for loss of $H_2O + CO$ (87.5 and 12.5%, respectively).

The CAD spectrum of the N-3 methylated isomer $\mathbf{2H}^+$ differed substantially from that of $\mathbf{1H}^+$ [Fig. 1(b)]. Ion $\mathbf{2H}^+$ showed loss of ammonia (*m/z* 153), CO_2 (*m/z* 126) and COOH (*m/z* 125) as important primary dissociations. The *m/z* 126 ion underwent further dissociations by loss of ammonia (*m/z* 109) and $CHNH_2$ (*m/z* 97). The differences in primary dissociations observed in the CAD spectra thus allowed unequivocal distinction of the methylation site in the imidazole ring, since the N-1 methylated isomer $\mathbf{1H}^+$ lost $H_2O + CO$, but the N-3 isomer did not.

CAD spectra obtained on the triple-quadrupole tandem mass spectrometer showed more extensive dissociations, including side-chain cleavages (*m/z* 95–97) and losses of substituents (*m/z* 81–83, 68) (Fig. 2). However, the characteristic loss of $H_2O + CO$ was dominant in the CAD spectrum of $\mathbf{1H}^+$ and virtually absent for $\mathbf{2H}^+$.

Similar results were obtained for several isomeric *N*-acetyldopamine-*N*-acetylhistidine conjugates and deacetylated derivatives, the latter isolated from acid hydrolyzates of insect



Scheme 1

CCC 1076–5174/98/040392–05 $17.50

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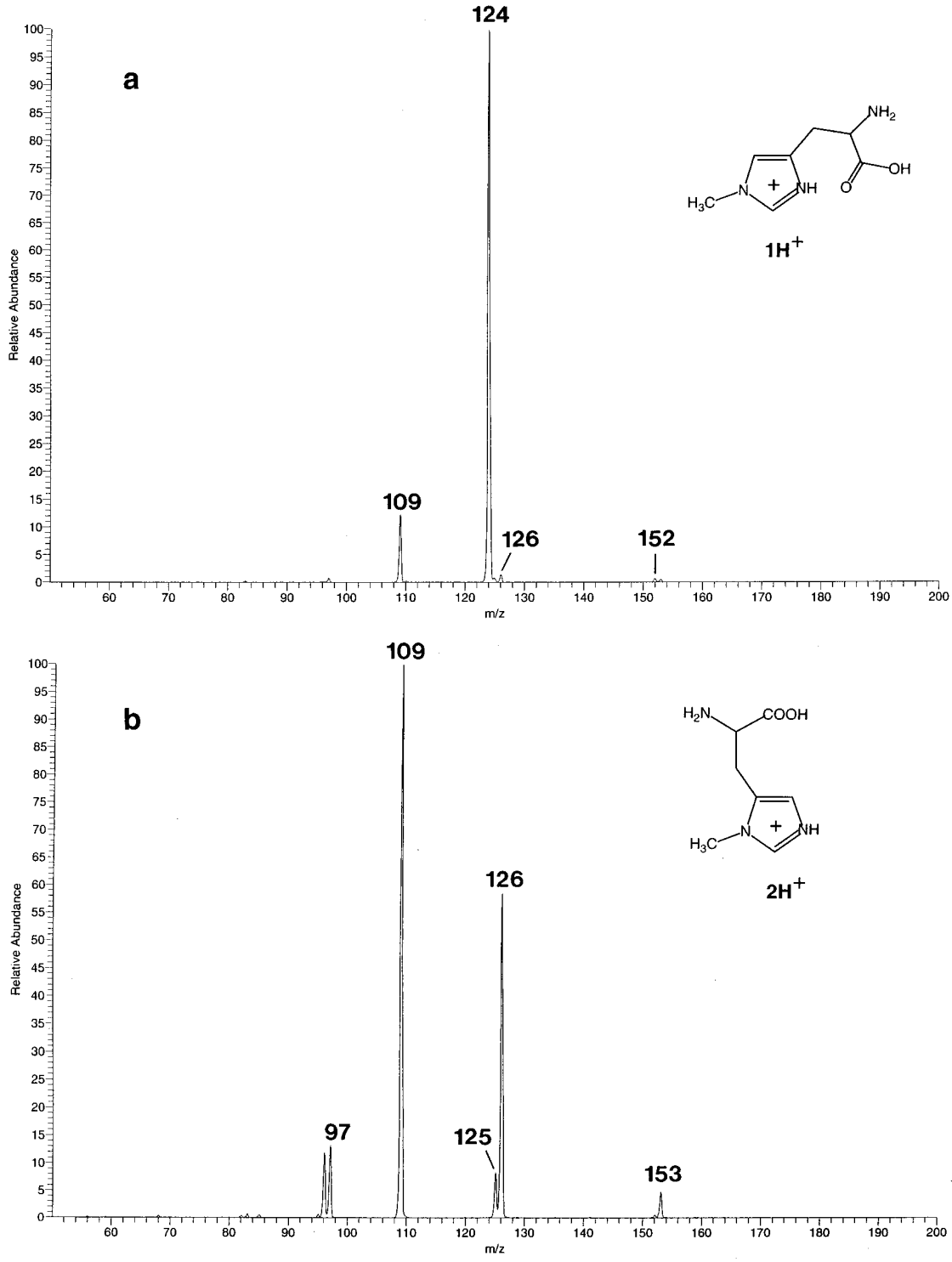

Figure 1. Electrospray ionization ion trap tandem mass spectra of (a) $\mathbf{1H}^+$ and (b) $\mathbf{2H}^+$.

cuticle.[6] Elimination of H_2O + CO apparently is also characteristic of the substituent position in *N*-alkyl-and *N*-aryl-substituted histidines.

To explain the different behaviors of gas-phase ions $\mathbf{1H}^+$ and $\mathbf{2H}^+$, we considered the energetics and reaction mechanisms of the dissociations. Eliminations of both CO_2 and H_2O + CO involve proton transfer, although it occurs in opposite directions. A proton is transferred from the carboxylic group on to a suitable receptor to trigger loss of CO_2 from $\mathbf{2H}^+$, whereas proton transfer on to the carboxylic hydroxyl group is necessary for elimination of H_2O from $\mathbf{1H}^+$. The imidazole ring is likely to contain the most basic sites in **1** and **2**, as deduced from the proton affinities (*PA*) of histidine (970 kJ mol^{-1})[7–9] and imidazole (935–941 kJ mol^{-1}),[10–13] compared with those of aliphatic amino acids (~910 kJ mol^{-1}).[8,9] Therefore, the question of the ring protonation site was addressed. We used density functional theory (DFT)[14] calculations with Becke's hybrid functional (B3LYP)[15] and a split-valence basis set [6–31G(d,p)][16] to obtain the energies, zero-point corrections and 298 K enthalpies for simpler model molecules 1-methylimidazole (**3**), 1,4-dimethylimidazole (**4**) and 1,5-dimethylimidazole (**5**) and the corresponding ions

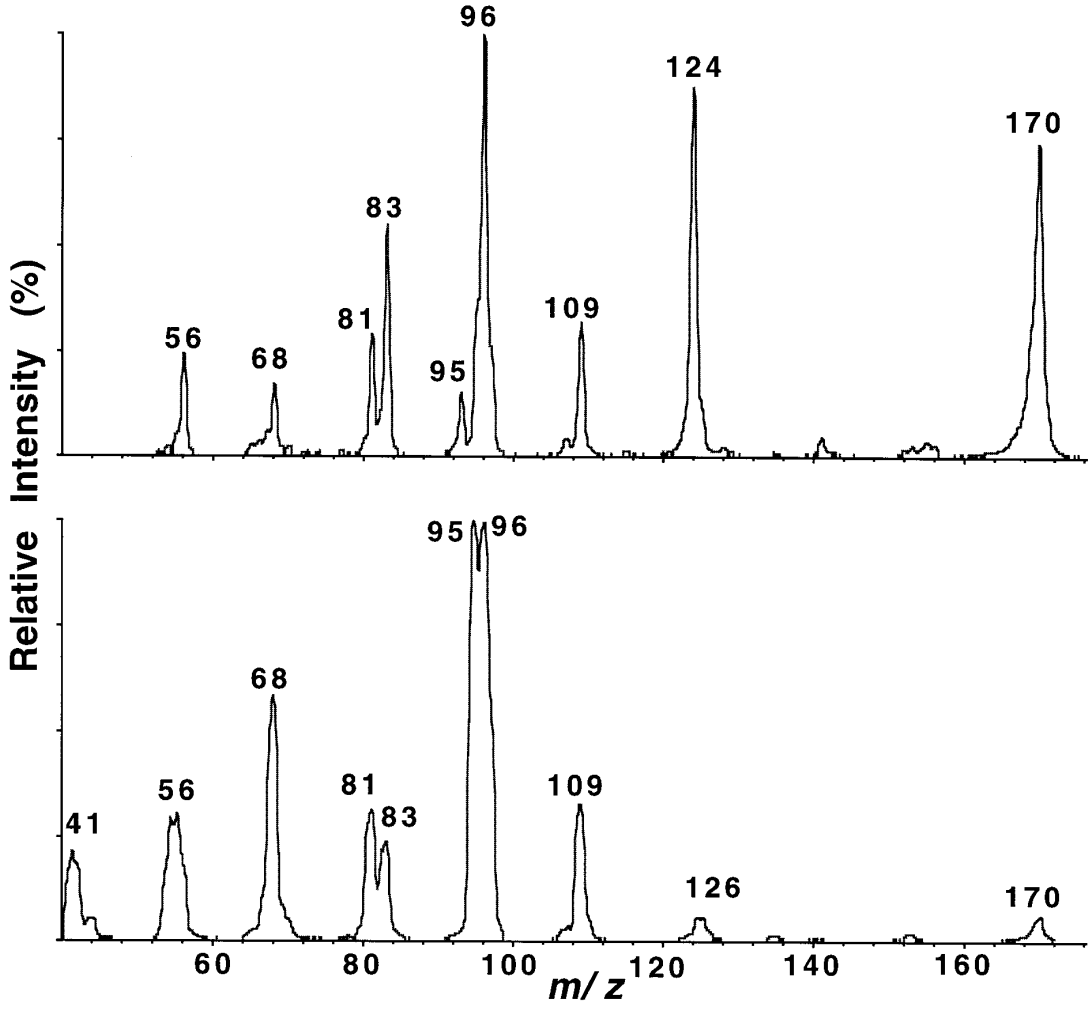

Figure 2. Electrospray ionization tandem quadrupole mass spectra of (top) $\mathbf{1H}^+$ and (bottom) $\mathbf{2H}^+$.

$\mathbf{3H}^+$, $\mathbf{4H}^+$ and $\mathbf{5H}^+$ (Table 1). Proton affinities calculated at this level of theory are usually accurate to within 20 kJ mol^{-1} of established experimental values.[17] Other protonated tautomers of **3**–**5** were considered to be much less stable[13] and, therefore, irrelevant under the mild protonation conditions of ESI-MS.[18] *N*-Methylation in **3** increased the proton affinity by ~50 kJ mol^{-1} compared with imidazole.[13] Further methylation at C-4 or C-5 had a smaller effect ($\Delta PA \approx 15$ kJ mol^{-1}) and the *PA*s of these isomers were indistinguishable at the present level of theory. By analogy, the presence of the substituents in the imidazole ring of **1** and **2** should direct protonation to the most basic, unsubstituted, imine nitrogen atom.

Structures for protonated *N*-methylhistidines $\mathbf{1H}^+$ and $\mathbf{2H}^+$ were obtained by semiempirical AM1[19] and PM3 calculations.[20] The calculated ion heats of formation are summarized in Fig. 3. The five lowest-energy conformers of $\mathbf{1H}^+$ ($\mathbf{1Ha}^+$-$\mathbf{1He}^+$) all showed intramolecular hydrogen bonds between the protonated ring nitrogen and the side-chain groups. Both AM1 and PM3 indicated that $\mathbf{1Ha}^+$ was the most stable isomer and had a hydrogen bond to the carboxylate carbonyl oxygen. The OH···H—N bonded isomer $\mathbf{1Hc}^+$

Table 1. Calculated energies and proton affinities

Species	Energy B3LYP/6–31G(d,p)[a]	ZPVE[b]	$H_{298} - H_0$[c]	*PA*[d]
1-Methylimidazole (**3**)	−265.536 285	250	17	990
1-Methyl-3*H*-imidazolium ($\mathbf{3H}^+$)	−265.924 513	285	17	
1,4-Dimethylimidazole (**4**)	−304.859 887	320	21	1005
1,4-Dimethyl-3*H*-imidazolium ($\mathbf{4H}^+$)	−305.254 196	355	22	
1,5-Dimethylimidazole (**5**)	−304.859 269	319	22	1006
1,5-Dimethyl-3*H*-imidazolium ($\mathbf{5H}^+$)	−305.253 502	355	21.5	

[a] In units of hartree; 1 hartree = 2625.5 kJ mol^{-1}.
[b] Zero-point vibrational energies in kJ mol^{-1} from B3LYP/6–31G(d,p) harmonic frequencies scaled by 0.96.
[c] 298 K enthalpy corrections in kJ mol^{-1}.
[d] Proton affinity at 298 K in kJ mol^{-1}.

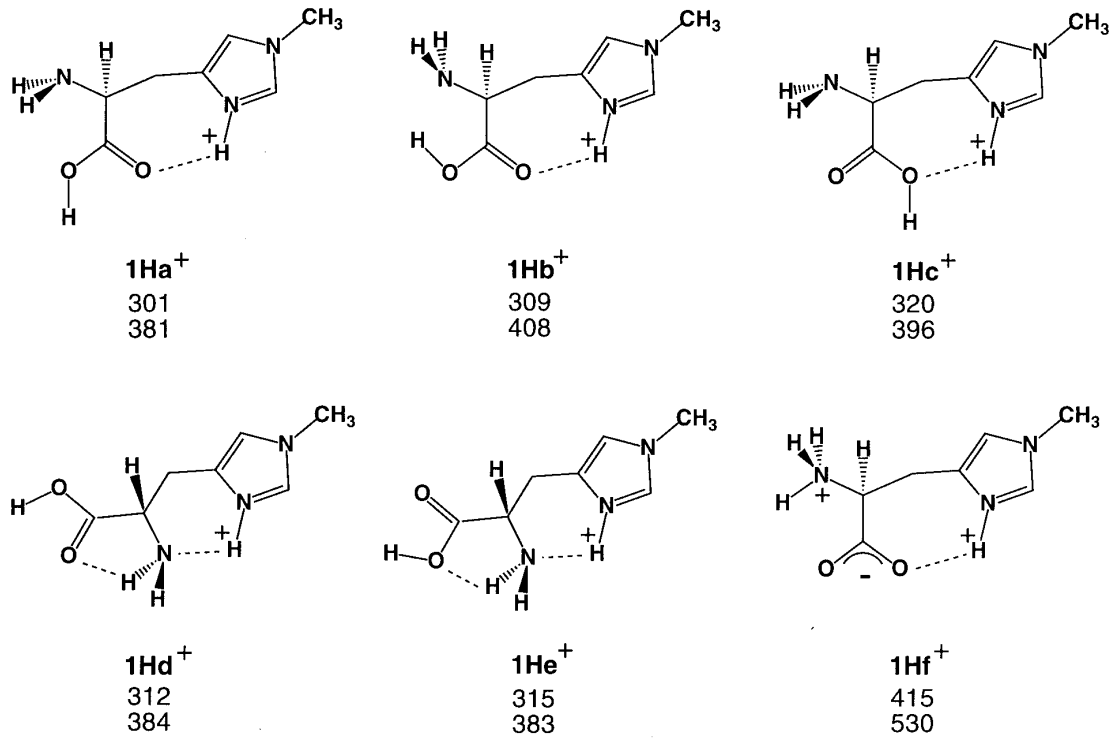

Figure 3. Optimized structures and heats of formation for $\mathbf{1H}^+$ isomers. Top values, AM1 enthalpies; bottom values, PM3 enthalpies.

was 15–20 kJ mol^{-1} less stable, whereas another C=O···HN isomer ($\mathbf{1Hb}^+$) and the NH_2···H—N bonded isomers $\mathbf{1Hd}^+$ and $\mathbf{1He}^+$ were intermediate in energy. In contrast, hydrogen bonding between the imidazole N—H bond and the side chain was sterically impossible in $\mathbf{2H}^+$ (Fig. 4). The four lowest-energy conformers, $\mathbf{2Ha}^+$–$\mathbf{2Hd}^+$, showed hydrogen bonding between the carboxylic and amino groups, but the protonated imidazole ring was not involved.

Structures $\mathbf{1Ha}^+$–$\mathbf{1He}^+$ are compatible with the CAD of ion $\mathbf{1H}^+$. Loss of water can occur by endothermic proton transfer from the imidazole nitrogen to the carboxylic OH group either directly in $\mathbf{1Hc}^+$ or by mediation by the amine group in $\mathbf{1He}^+$. We presume that the barriers to conformational interconversions in $\mathbf{1Ha}^+$–$\mathbf{1He}^+$ are lower than the dissociation threshold. Elimination of water is followed by rapid loss of CO, according to the mechanism suggested by Harri-

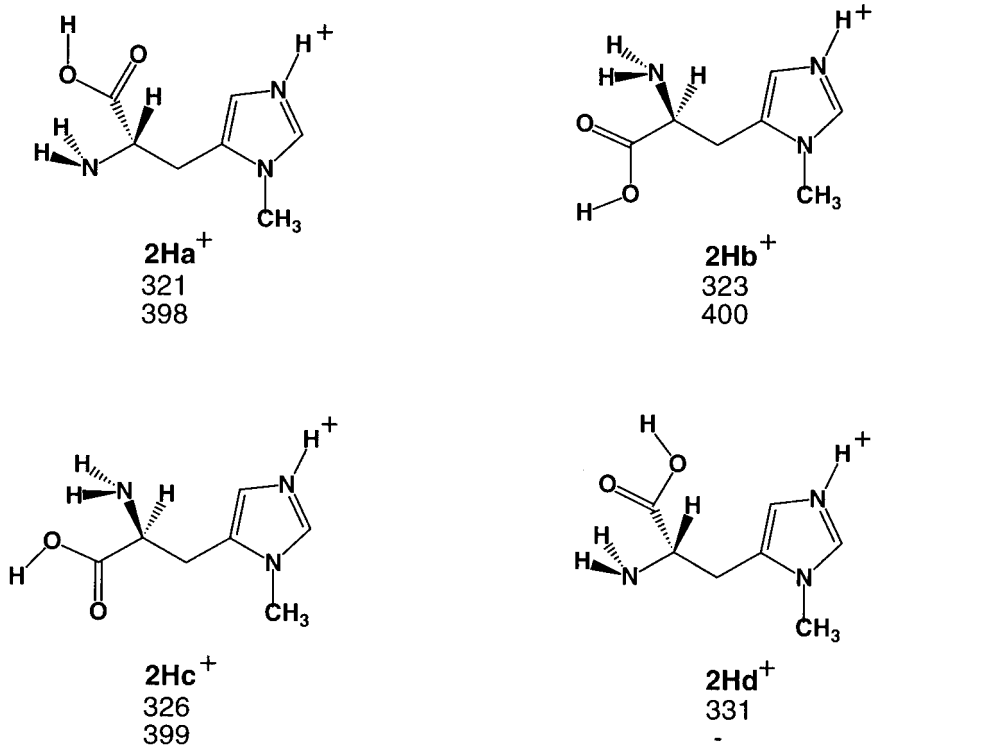

Figure 4. Optimized structures and heats of formation for $\mathbf{2H}^+$ isomers. Top values, AM1 enthalpies; bottom values, PM3 enthalpies.

son and co-workers.[21] The dominant loss of $H_2O + CO$ from $\mathbf{1H}^+$ resembles the analogous dissociation of protonated histidine,[22] $HisH^+$, which also occurs predominantly on CAD of $HisH^+$ prepared by electrospray ionization (spectrum not shown).

The reverse transfer of the proton from the carboxylic group to the imidazole ring to trigger decarboxylation is hampered by the low basicity of the ring nitrogens due to protonation. Elimination of CO_2 from $\mathbf{1H}^+$ and $\mathbf{2H}^+$ may occur by transfer of the carboxylic proton to the amine group to form zwitterionic intermediates.[23] For $\mathbf{1H}^+$, both AM1 and PM3 located a high-energy, albeit stable, intermediate, $\mathbf{1Hf}^+$, which was a local energy minimum characterized by harmonic frequency analysis. Structure $\mathbf{1Hf}^+$ had a remarkably long $C{-}CO_2$ bond (1.62 Å) and showed hydrogen bonding between the carboxylate group and the histidine proton (Fig. 3). In contrast, attempted geometry optimizations of zwitterionic structures derived from $\mathbf{2H}^+$ resulted in dissociation of the $C{-}CO_2$ bond and elimination of CO_2. Apparently, the $CO_2^- \cdot\cdot H^+N$ hydrogen bonding in $\mathbf{1Hf}^+$ is needed to stabilize the zwitterionic structure.

In summary, proton transfer from and on to the carboxylic group in protonated *N*-substituted histidines initiates dissociations of gas-phase ions that allow unequivocal distinction of positional isomers by ESI-MS/MS. Isomer differentiation by ESI-MS/MS can be achieved with small quantities of impure samples, which are not amenable to NMR analysis. Further applications of this facile methodology to protein–catecholamine conjugates present in insect cuticle will be reported in a forthcoming full paper.

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Yours,

F. TURECEK,[1]* J. L. KERWIN,[2] R. XU[3] and K. J. KRAMER[4]

[1] Department of Chemistry,
Bagley Hall,
Box 351700,
University of Washington,
Seattle,
Washington 98195,
USA

[2] Department of Botany,
University of Washington,
Seattle,
Washington 98195,
USA

[3] Department of Entomology,
Kansas State University,
Manhattan,
Kansas 66506,
USA

[4] Grain Marketing and Production Research Center,
Agricultural Research Service,
United States Department of Agriculture,
Manhattan,
Kansas 66502,
USA

* *Correspondence to*: F. Turecek, Department of Chemistry, Bagley Hall, Box 351700, University of Washington, Seattle, Washington 98195, USA
E-mail: turecek@macmail.chem.washington.edu

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