Isomeric Differentiation and Acidic Metabolite Identification by Piperidine-based Tagging, LC-MS/MS, and Understanding of the Dissociation Chemistries: Supporting Information

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Table of content

Figure S1. Reaction scheme for tagging of citrate with N-(4-aminophenyl)piperidine.

Figure S2. RIC ion chromatograms for (top panel) untagged, deprotonated citrate and isocitrate; (bottom panel) triply tagged, triply protonated citrate and isocitrate. The magnification of the top panel image is increased for ease of viewing.

Figure S3. Global minimum triply tagged, triply protonated (a) citrate and (b) isocitrate. The approximate positions of protonation have indicated with black ε for ease of viewing.

Figure S4. Rate-limiting transition structure of triply tagged, triply protonated citrate, C_{39}H_{53}N_{6}O_{4}^{3+}, m/z 223 to form C_{26}H_{34}N_{4}O_{3}^{2+}, m/z 225, and C_{13}H_{19}N_{2}O^{+}, m/z 219:

(C1) \ 223 (z=3) \rightarrow \ 225 (z=2) + \ 219 (z=1)

Figure S5. Rate-limiting transition structure of triply tagged, triply protonated isocitrate, C_{39}H_{53}N_{6}O_{4}^{3+}, m/z 223 to form C_{26}H_{36}N_{4}O_{2}^{2+}, m/z 218 and C_{13}H_{17}N_{2}O_{2}^{+}, m/z 233:

(I1) \ 223 (z=3) \rightarrow \ 233 (z=1) + \ 218 (z=2)

Figure S6. Rate-limiting transition structure of triply tagged, triply protonated citrate, C_{39}H_{53}N_{6}O_{4}^{3+}, m/z 223 to form C_{27}H_{36}N_{4}O_{3}^{2+}, m/z 232, and C_{11}H_{17}N_{2}^{+}, m/z 177:

(C2) \ 223 (z=3) \rightarrow \ 232 (z=2) + \ CO + \ 177 (z=1)

Figure S7. Rate-limiting transition structure of triply tagged, triply protonated isocitrate, C_{39}H_{53}N_{6}O_{4}^{3+}, m/z 223 to form C_{27}H_{36}N_{4}O_{3}^{2+}, m/z 232, and C_{11}H_{17}N_{2}^{+}, m/z 177:

(I2) \ 223 (z=3) \rightarrow \ 232 (z=2) + \ CO + \ 177 (z=1)
Figure S8. (Top panel) Consecutive fragmentation transition structure of C_{26}H_{34}N_{4}O_{5}^{2+}, m/z 225 to form C_{12}H_{13}N_{2}O^{+}, m/z 203, and C_{14}H_{19}N_{2}O^{+}, m/z 247; (Lower panel) Transition structure (≥71 kJ mol\(^{-1}\)) which is substantially lower than the preceding TS (166 kJ mol\(^{-1}\)) which thus enables the process to occur spontaneously: (C3) \(225 (z=2) \rightarrow 247 (z=1) + 203 (z=1)\). The fundamental reason underpinning the low consecutive barrier is the systematic reduction in the charge-charge repulsion throughout the dissociation processes.

Figure S9. (Top panel) Consecutive fragmentation transition structure of C_{26}H_{34}N_{4}O_{5}^{2+}, m/z 225 to form C_{13}H_{17}N_{2}O_{3}^{+}, m/z 273, and C_{11}H_{17}N_{2}^{+}, m/z 177; (Middle panel) Complex H\(^{+}\) transfer from the amide nitrogen to oxygen to form an iminol structure (TS≥163 kJ mol\(^{-1}\)). This process is concerted and involves a substantial rotation with the H\(^{+}\) transfer catalyzed by the adjacent carbonyl oxygen. (Lower panel) TS (≥188 kJ mol\(^{-1}\)) which thus enables the cyclization and amide bond cleavage process to occur: (C4) \(225 (z=2) \rightarrow 273 (z=1) + 177 (z=1)\). This reaction is entropically favored (\(\Delta S^{e} = 182 J K^{-1} mol^{-1}\)) as a consecutive process which also serves to reduce the charge-charge repulsion experienced by the analyte fragments.

Figure S10. (Top panel) Consecutive fragmentation transition structure of C_{27}H_{36}N_{4}O_{3}^{2+}, m/z 232 to form C_{12}H_{15}N_{2}O^{+}, m/z 203 and C_{13}H_{21}N_{2}O_{2}^{+}, m/z 261; (Lower panel) TS (42 kJ mol\(^{-1}\)) which is substantially lower than the preceding TS (213 kJ mol\(^{-1}\)) so the process can occur spontaneously provided sufficient time is available to access the TS: (C5) \(232 (z=2) \rightarrow 261 (z=1) + 203 (z=1)\). Again, the fundamental reason underpinning the low consecutive barrier is the systematic reduction in the charge-charge repulsion throughout these dissociation processes.

Figure S11. (Top panel) Consecutive fragmentation of C_{26}H_{36}N_{4}O_{2}^{2+}, m/z 218, to form C_{15}H_{19}N_{2}O_{2}^{+}, m/z 259, and C_{11}H_{17}N_{2}^{+}, m/z 177; (Lower panel) TS (152 kJ mol\(^{-1}\)) which is substantially lower than the preceding TS ((I1) 183 kJ mol\(^{-1}\)) so the process can occur spontaneously provided sufficient time is available to access the TS:

\[I6 \quad 218 (z=2) \rightarrow 259 (z=1) + 177 (z=1)\]

Figure S12. Isocitrate: (Top panel) Concerted loss of 2 ethene molecules from the piperidine tag located furthest from the hydroxyl group to form the triply charged peak at m/z 204.449.; (Lower, left panel) Initial C-C and C-N bond cleavage generating a dimer of ethene and a primary carbocation as part of the triply charged product of identical m/z to the precursor ion (TS ≥ 206 kJ mol\(^{-1}\)); (Lower, right panel) Rate-limiting dissociation of a second C-C bond to form a second ethene molecule and protonated imine group from the formerly piperidine functional group (TS ≥ 269 kJ mol\(^{-1}\)). The newly formed product has fewer electrons to stabilize the three charges with so is prone to subsequent dissociation (see main text).

\[(I3-CNH side) \quad 223 (z=3) \rightarrow [223 \text{ dimer (z=3)}] \text{ (step 1)}\]

\[(I3-CNH side) \quad [223 \text{ dimer (z=3)}] \rightarrow 204.4 \ (z=3) + C_{2}H_{4} + C_{2}H_{4} \text{ (step 2)}\]

Figure S13. Isocitrate: This consecutive reaction is essentially identical to the direct reaction shown in Scheme 1b, and Figure S4 ((I1) 223 (z=3) → 233 (z=1) + 218 (z=2)). The major difference is the precursor ion has lost 2 ethene molecules beforehand which makes subsequent dissociations very entropically favorable (407.5 J K\(^{-1}\) mol\(^{-1}\)) which in turn reduces \(\Delta G\) (Table S2).
(I4) $204.4 \ (z=3) \rightarrow 190 \ (z=2) + 233 \ (z=1) \ (C_2H_4 \ loss \ from \ CNH \ side)$

**Figure S14.** Fragmentation of doubly tagged, doubly protonated malate. Precursor ions of m/z 226.13, C$_{26}$H$_{36}$N$_4$O$_{3}^{2+}$, were subjected to LC-MS/MS fragmentation.

**Figure S15.** Global minimum doubly tagged, doubly protonated malate.

**Figure S16.** (Top panel) Primary mechanism of doubly tagged, doubly protonated malate (C$_{26}$H$_{36}$N$_4$O$_{3}^{2+}$, m/z 226), fragmentation to form the alkene-ol terminated C$_{13}$H$_{19}$N$_2$O$_1^+$, m/z 219 structure, and aldehyde-terminated C$_{13}$H$_{17}$N$_2$O$_2^+$, m/z 233 peaks; (Lower panel) Rate-limiting transition structure of doubly tagged, doubly protonated malate, C$_{26}$H$_{36}$N$_4$O$_{3}^{2+}$, m/z 226 to form the dominant C$_{13}$H$_{19}$N$_2$O$_1^+$, m/z 219, and C$_{13}$H$_{17}$N$_2$O$_2^+$, m/z 233 peaks:

(M1) $226 \ (z=2) \rightarrow 219 \ (z=1) + 233 \ (z=1)$

**Figure S17.** Doubly tagged, doubly protonated malate dissociation. (Top panel) Loss of C$_4$H$_8$ from the piperidine tag located furthest from the hydroxyl group to form the doubly charged peak at m/z 198.11, C$_{22}$H$_{36}$N$_4$O$_3^{2+}$; (Lower panels) Key transition structures from the mechanism described above. (M2) $226 \ (z=2) \rightarrow 219 \ (z=1) + CO + 177 \ (z=1)$

**Figure S18.** Doubly tagged, doubly protonated malate dissociation. (Top panel) Loss of C$_4$H$_8$ from the piperidine tag located furthest from the hydroxyl group to form the triply charged peak at m/z 198.11; (Lower panels) Key consecutive transition structures from the mechanism described above. This mechanism is entropically favorable (Table S4), enabling it to be competitive with the other reactions: (M3) $226 \ (z=2) \rightarrow 198 \ (z=2) + C_4H_8$

**Figure S19.** Pseudo-MS$^3$ fragmentation of the m/z 225 ion population generated from triply tagged, triply protonated citrate.

**Figure S20.** Pseudo-MS3 fragmentation of the m/z 219.1 ion population generated from triply tagged, triply protonated citrate.

**Figure S21.** MS$^3$ fragmentation of the m/z 232 ion population generated from triply tagged, triply protonated citrate.

**Figure S22.** Pseudo-MS$^3$ fragmentation of the m/z 177.14 ion population generated from triply tagged, triply protonated citrate.

**Figure S23.** Pseudo-MS$^3$ fragmentation of the m/z 273.1 ion population generated from triply tagged, triply protonated citrate.

**Figure S24.** Pseudo-MS$^3$ fragmentation of the m/z 203.1 ion population generated from triply tagged, triply protonated citrate.

**Figure S25.** MS$^3$ fragmentation of the m/z 247.1 ion population generated from triply tagged, triply protonated citrate.

**Figure S26.** MS$^3$ fragmentation of the m/z 261.1 ion population generated from triply tagged, triply protonated citrate.
**Figure S27.** Pseudo-MS$^3$ fragmentation of the m/z 233.1 ion population generated from triply tagged, triply protonated isocitrate.

**Figure S28.** Pseudo-MS$^3$ fragmentation of the m/z 218.1 ion population generated from triply tagged, triply protonated isocitrate.

**Figure S29.** MS$^3$ fragmentation of the m/z 190.1 ion population generated from triply tagged, triply protonated isocitrate.

**Figure S30.** MS$^3$ fragmentation of the low abundance m/z 225.1 ion population generated from triply tagged, triply protonated isocitrate. Note obvious differences to the substantial m/z 225 ion peak from citrate (Figure S19).

**Figure S31.** Example MS/MS of deuterated triply tagged, triply charged citrate, C$_{39}$H$_{46}$D$_{7}$N$_{6}$O$_{4}$$^{3+}$, m/z 225.49. See Table S3 for detailed summaries specific m/z shifts.

**Figure S32.** Example MS/MS of deuterated triply tagged, triply charged isocitrate, C$_{39}$H$_{46}$D$_{7}$N$_{6}$O$_{4}$$^{3+}$, m/z 225.49. See Table S3 for detailed summaries specific m/z shifts.

**Figure S33.** Crude breakdown graphs as a function of HCD value for triply tagged Citrate and Isocitrate. Isocitrate is more difficult to fragment initially but has a steeper slope with increasing HCD value.

**Table S1:** Relative energies of the rate-limiting transition structures of triply tagged, triply protonated citrate, C$_{39}$H$_{53}$N$_{6}$O$_{4}$$^{3+}$, m/z 223.

**Table S2:** Relative energies of rate-limiting transition structures of triply tagged, triply protonated isocitrate, C$_{39}$H$_{53}$N$_{6}$O$_{4}$$^{3+}$, m/z 223.

**Table S3:** Summarized shifts in m/z of the various fragments of triply tagged, triply protonated citrate and isocitrate generated from solutions of D$_2$O/acetonitrile.

**Table S4:** Relative energies of major rate-limiting transition structures of doubly tagged, doubly protonated malate (C$_{26}$H$_{36}$N$_{4}$O$_{3}$$^{2+}$, m/z 226.1).
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\[ (\text{C1}) \text{223 (z=3)} \rightarrow \text{225 (z=2)} + \text{219 (z=1)} \]
Figure S5. Rate-limiting transition structure of triply tagged, triply protonated isocitrate, $C_{39}H_{53}N_6O_4^{3+}$, m/z 223 to form $C_{26}H_{36}N_4O_2^{2+}$, m/z 218 and $C_{13}H_{17}N_2O_2^+$, m/z 233:

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\[
(16) \quad 218 \text{ (z=2)} \rightarrow 259 \text{ (z=1)} + 177 \text{ (z=1)}
\]
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\[
(I3-CN\text{H side}) \quad 223 \text{ (z=3)} \rightarrow [223 \text{ dimer (z=3)}] \text{ (step 1)}
\]

\[
(I3-CN\text{H side}) \quad [223 \text{ dimer (z=3)}] \rightarrow 204.4 \text{ (z=3)} + C_2H_4 + C_2H_4 \text{ (step 2)}
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Figure S24. Pseudo-MS$^3$ fragmentation of the m/z 203.1 ion population generated from triply tagged, triply protonated citrate.
Figure S25. MS$^3$ fragmentation of the m/z 247.1 ion population generated from triply tagged, triply protonated citrate.
Figure S26. MS\textsuperscript{3} fragmentation of the m/z 261.1 ion population generated from triply tagged, triply protonated citrate.
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Table S2: Relative energies of rate-limiting transition structures of triply tagged, triply protonated isocitrate, C$_{39}$H$_{53}$N$_{6}$O$_{4}$$^{3+}$, m/z 223.
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<td>C\textsubscript{39}H\textsubscript{46}D\textsubscript{7}N\textsubscript{6}O\textsubscript{4}3\textsuperscript{+}</td>
<td>223.1</td>
<td>C\textsubscript{39}H\textsubscript{53}N\textsubscript{6}O\textsubscript{4}3\textsuperscript{+}</td>
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<td>Citrate</td>
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<td>C\textsubscript{13}H\textsubscript{16}D\textsubscript{5}N\textsubscript{5}O\textsubscript{1}+</td>
<td>219.1</td>
<td>C\textsubscript{13}H\textsubscript{19}N\textsubscript{5}O\textsubscript{1}+</td>
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<td>227.1</td>
<td>C\textsubscript{23}H\textsubscript{30}D\textsubscript{2}N\textsubscript{4}O\textsubscript{2}2\textsuperscript{+}</td>
<td>225.1</td>
<td>C\textsubscript{23}H\textsubscript{34}N\textsubscript{4}O\textsubscript{2}2\textsuperscript{+}</td>
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<td>234.1</td>
<td>C\textsubscript{27}H\textsubscript{32}D\textsubscript{4}N\textsubscript{4}O\textsubscript{3}2\textsuperscript{+}</td>
<td>232.1</td>
<td>C\textsubscript{27}H\textsubscript{36}N\textsubscript{4}O\textsubscript{3}2\textsuperscript{+}</td>
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<tr>
<td>Citrate</td>
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<td>C\textsubscript{11}H\textsubscript{14}D\textsubscript{3}N\textsubscript{2}+</td>
<td>177.1</td>
<td>C\textsubscript{11}H\textsubscript{17}N\textsubscript{2}+</td>
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<td>Citrate</td>
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<td>C\textsubscript{12}H\textsubscript{14}D\textsubscript{1}N\textsubscript{2}O\textsubscript{1}+</td>
<td>203.1</td>
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<td>C\textsubscript{15}H\textsubscript{17}N\textsubscript{2}O\textsubscript{3}+</td>
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<td>247.1</td>
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<td>C\textsubscript{39}H\textsubscript{53}N\textsubscript{6}O\textsubscript{4}3\textsuperscript{+}</td>
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<td>177.1</td>
<td>C\textsubscript{11}H\textsubscript{17}N\textsubscript{2}+</td>
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Table S3: Summarized shifts in m/z of the various fragments of triply tagged, triply protonated citrate and isocitrate generated from solutions of D\textsubscript{2}O/acetonitrile.
Table S4: Relative energies of major rate-limiting transition structures of doubly tagged, doubly protonated malate (C_{26}H_{36}N_{4}O_{3}^{2+}, m/z 226.1).

<table>
<thead>
<tr>
<th></th>
<th>$E_{el}$/H</th>
<th>$E_{el+ZPE}$/H</th>
<th>$\Delta E_{el+ZPE,0K}$/kJ mol$^{-1}$</th>
<th>$\Delta H_{298K}$/kJ mol$^{-1}$</th>
<th>$\Delta G_{298K}$/kJ mol$^{-1}$</th>
<th>$\Delta S_{298K}$/JK$^{-1}$ mol$^{-1}$</th>
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