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RESEARCH ARTICLE

Investigation of Fragmentation of Tryptophan Nitrogen Radical Cation

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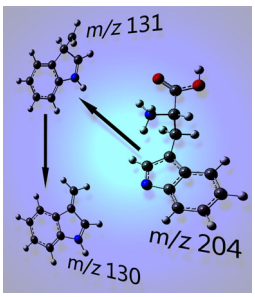

Abstract. This work describes investigation of the fragmentation mechanism of tryptophan *N*-indolyl radical cation, H_3N^+-TrpN$^\bullet$ (*m/z* 204) studied via DFT calculations and several gas-phase experimental techniques. The main fragment ion at *m/z* 131, shown to be a mixture of up to four isomers including 3-methylindole (3MI) π-radical cation, was found to undergo further loss of an H atom to yield one of the two isomeric *m/z* 130 ions. 3-Methylindole radical cation generated independently (via CID of $[Cu^{II}(terpy)3MI]^{\bullet 2+}$) displayed gas-phase reactivity partially similar to that of the *m/z* 131 fragment, further confirming our proposed mechanism. CID of deuterated tryptophan *N*-indolyl radical cation (*m/z* 208) suggested that up to six H atoms are involved in the pathway to formation of the *m/z* 131 ion, consistent with hydrogen atom scrambling during CID of protonated Trp.

Keywords: Tryptophan N-indole radical, 3-Methylindole, Quinolinium ion, Collision-induced dissociation, Ion-molecule reactions, Density functional theory calculations

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Introduction

Radical ions of proteins, peptides, and amino acids have attracted considerable interest in the past decade [1–4]. Understanding fragmentation mechanisms of peptide radical ions is crucial for interpreting experimental data in techniques like electron capture dissociation [5], electron transfer dissociation [6], and radical-directed dissociation [7]. Among the various amino acid-based radicals, tryptophan is especially intriguing as it can form two types of biologically important radicals—the tryptophan π-radical cation (Trp$^{\bullet+}$) and the indolyl radical (TrpN$^\bullet$). Both types are believed to be involved in electron transfer processes [8] as well as in protein oxidation [9]. Both types of Trp radicals were studied by tandem MS in peptide systems [10–13].

In a recent work, we explored gas-phase properties of two isomeric, regiospecifically formed tryptophan-based radical cations [14]. We examined and compared such properties as their reactivities, infrared spectra, structure and energetics, and CID fragmentation patterns. Fragmentation of the tryptophan π-radical cation formed via CID of copper (II) ternary complexes had only one major side chain-based fragment ion, 3-methyleneindolium (*m/z* 130), and its structure and the mechanism of formation has been described previously [15, 16]. The tryptophan *N*-indolyl radical cation was generated via CID of *N*-nitrosylated protonated tryptophan (Equation 1) as described by others [13, 17]:

$$H_3N^+-TrpNH \xrightarrow{X-NO} H_3N^+-TrpN-NO \rightarrow H_3N^+-TrpN^\bullet \quad (1)$$

When formed, this distonic ion formally has the spin localized in a sigma orbital on the N of the indole ring, but in reality it is delocalized over the π-system of the indole [14].

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The CID fragmentation of H_3N^+-TrpN$^\bullet$ exhibits completely different neutral losses compared with the π-radical cation, which are also different from those of the protonated tryptophan [18]. The major product of CID of the tryptophan *N*-indolyl radical cation (*m*/*z* 204) is the ion at *m*/*z* 131 (see Figure 1 of Reference [14] or Figure 3 inset below). Although the minor fragments were all assigned in our previous work [14], questions remain with regard to the structure of the ion at *m*/*z* 131 (which we tentatively assigned as 3-methylindole radical cation, 3MI$^{\bullet+}$) and the mechanism by which it is formed. 3-Methylindole is a known degradation product of tryptophan that was implicated in pulmonary toxicity in animals [19]. The mechanism of 3MI formation does not involve tryptophan radicals, but proceeds through enzymatic deamination and decarboxylation of tryptophan leading to indole-3-acetic acid [20], which in turn gets oxidized to its radical cation, decarboxylation of which leads to 3MI radicals. Some studies attributed toxicity of 3MI to its reactive radical intermediates including 3MI$^{\bullet+}$ [21]. While our gas-phase fragmentation occurs via a different pathway, it does suggest a way for 3MI$^{\bullet+}$ production from tryptophan *N*-indolyl radical. In this work, we use a combination of gas-phase experimental and theoretical techniques to shed light on the structure of *m*/*z* 131 ions and the pathways leading to their formation.

Experimental

Chemicals and Reagents

All chemicals and reagents were used as received without any further purification. L-tryptophan, 3-methylindole, 2,2';6',2"-terpyridine (terpy), and *tert*-butyl nitrite, di-*tert*-butyl nitroxide were all purchased from Sigma-Aldrich (Milwaukee, WI, USA). Methanol and D_2O were purchased from Fisher (Pittsburg, PA, USA). Water was purified (18 MΩ) in-house.

Mass Spectrometry

Mass spectrometry experiments were carried out at Northern Illinois University using a Bruker Esquire 3000 quadrupole ion trap mass spectrometer (Bruker Daltonics, Billerica, MA, USA) modified to conduct ion-molecule reactions as described previously [22, 23]. The *N*-nitrosylated tryptophan was generated by allowing a 1.5:1 mixture of *tert*-butylnitrite and a 1 mM solution of Trp (in 50/50 methanol/water with 1% acetic acid) to react for 10 min at room temperature. The reaction mixture was diluted a hundredfold using 50/50 methanol/water and introduced into the ESI source of the mass spectrometer at a flow rate of 4 μL min^{-1}. The nebulizer gas, needle voltage, and temperature were adjusted to about 15 psi, 3.4 kV, and 200°C, respectively. Radical cations H_3N^+-TrpN$^\bullet$ were produced by CID using collision energy sufficient to dissociate the majority of the precursor ions. The deuterated form of the radical was formed using the same procedure, except with D_2O/CH_3OD as the solvent.

For the production of 3-methylindole (3MI) π-radical cation, 200 μL of 1 mM 3MI stock solution (in 50/50 methanol/water) was mixed with 100 μL of 1:1 mixture of 1 mM $CuSO_4$ and 2,2';6,2"-terpyridine stock solutions. The mixture was then diluted with 700 μL of methanol and immediately introduced into the ESI source of the mass spectrometer at a flow rate of 4 μL min^{-1}. The nebulizer gas, needle voltage, and temperature were adjusted to 18 psi, 3.4 kV, and 200°C. The radical was obtained through the fragmentation of [Cu^{II}(terpy)3MI]$^{\bullet2+}$ ternary complex using collision-induced dissociation. The corresponding radical cations were then mass-selected with a window of 1*m*/*z* for further analysis.

Computational

All calculations were carried out by the Gaussian09 package of programs [24]. Geometry optimizations and harmonic vibrational frequency calculations were performed at B3LYP/6-311++G(d,p) level of theory [25–31]. In the case of open-shell systems, spin-unrestricted calculations (UB3LYP) were used. Intrinsic reaction coordinate (IRC) calculations [32] were employed on all the transition states, followed by geometry optimizations on the structures produced by the IRC calculations in order to ensure that the transition states were indeed connected to the appropriate reactant and product ions.

Results and Discussion

Theoretical Calculations

In our previous work, we calculated the barrier for the *N*-indolyl tryptophan radical cation (H_3N^+-TrpN$^\bullet$) rearranging into the lower energy π-radical cation to be 137 kJ mol^{-1} [14]. Since the π-radical cation fragments under CID conditions to give the 3-methylindoleum ion at *m*/*z* 130 and there is very little *m*/*z* 130 present in the CID of H_3N^+-TrpN$^\bullet$, the fragmentation pathway must have a barrier that is lower than required for conversion into the π-radical. Earlier, we postulated that the base peak in the CID spectrum of H_3N^+-TrpN$^\bullet$, *m*/*z* 131, is the radical cation of 3-methylindole (3MI$^{\bullet+}$). In order to form this species, the initial ion at *m*/*z* 204 has to lose CO_2 and NH=CH_2 moieties. The process is initiated by a proton transfer from the NH_3^+ to C3 of the indole (Figure 1a). After a series of geometry changes through bond rotations (first about C_β–$C\gamma$, then C_α–C_β and C–OH bonds, see Supplementary Figure S1 for details) the C_α–C bond cleaves followed by HAT from the C-terminus to the indole nitrogen atom resulting in the loss of CO_2. This step of the mechanism is consistent with CO_2 loss reported earlier by Knudsen and Julian [13] for C-terminal TrpN$^\bullet$ in small peptides. After another hydrogen-atom transfer (HAT) from the N-terminus to C_α followed by C_α–C_β bond dissociation, an isomer of the *m*/*z* 131 ion (**1**) is formed with an overall barrier of 129 kJ mol^{-1}, slightly lower than that required for the formation of the π-radical cation by proton transfer (137 kJ mol^{-1}). This higher energy isomer **1** of the *m*/*z* 131 ion can undergo a further 1,2-HAT to yield the lower energy

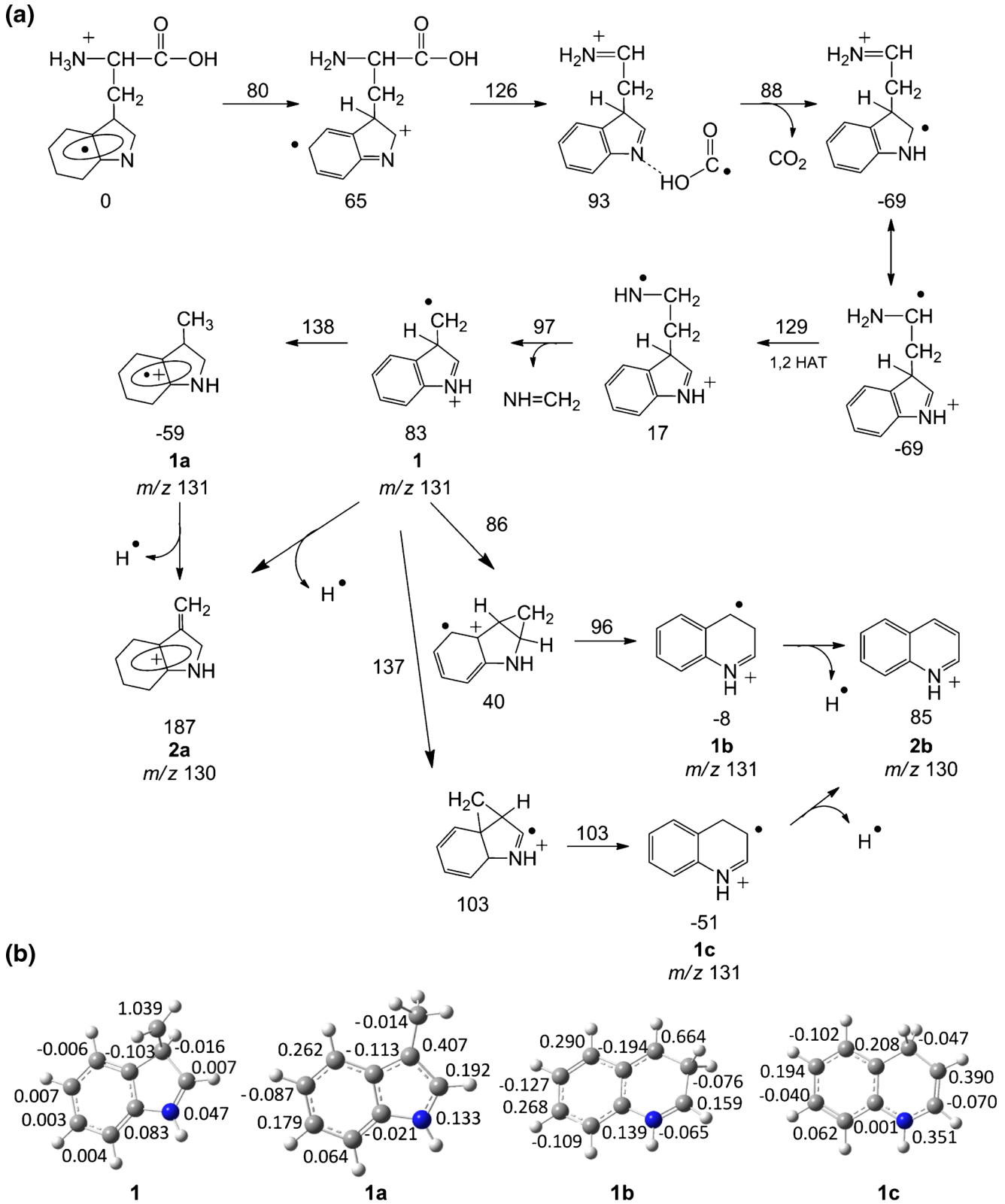


Fig. 1. (**a**) Tryptophan N-indole radical fragmentation path calculations. (The enthalpies (ΔH°_0) are relative to tryptophan nitrogen radical H_3N^+-TrpN$^{\bullet}$; all energies are in kJ mol^{-1}). (**b**) Spin densities of heavy atoms in m/z 131 isomers calculated at the B3LYP/6-311++G(d,p) level

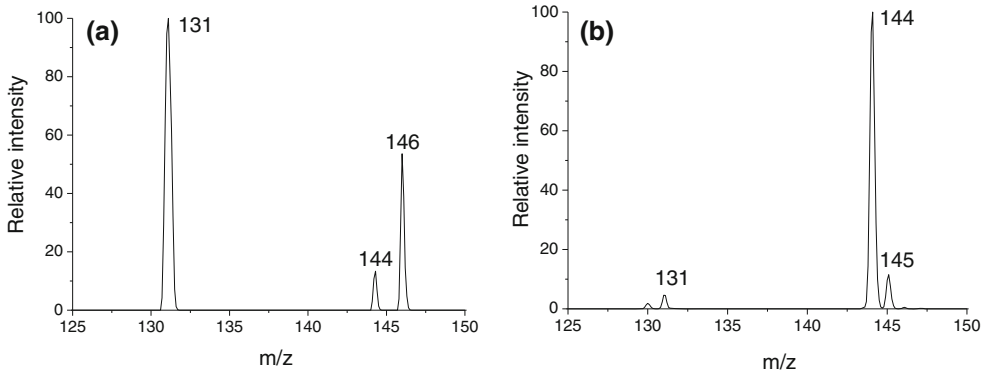


Fig. 2. Ion-molecule reactions of di-*tert*-butyl nitroxide with *m/z* 131 ions: (**a**) obtained via CID of H_3N^+-TrpN$^\bullet$; (**b**) 3MI$^{\bullet+}$ obtained via CID of [Cu^{II}(terpy)(3MI)]$^{\bullet 2+}$ (pulse duration 350 μs; reaction time 2000 ms)

structure, the 3MI$^{\bullet+}$ ion (**1a**). Both *m/z* 131 ions, **1** and **1a**, can lose a hydrogen atom and form 3-methyleneindolium ion (*m/z* 130, **2a**). Alternatively, ion **1** can undergo ring expansions leading to lower energy isomers with *m/z* 131 **1b** and **1c**, either of which can lose a hydrogen atom and form quinolinium ion **2b** at *m/z* 130. The overall barriers associated with forming **1a**, **1b**, and **1c** from **1** are comparable suggesting that a mixture of isomers can be formed upon CID. The structures of the isomeric *m/z* 131 ions **1**-**1c** displaying spin delocalization are shown in Figure 1b.

Collision-Induced Dissociation and Ion-Molecule Reactions

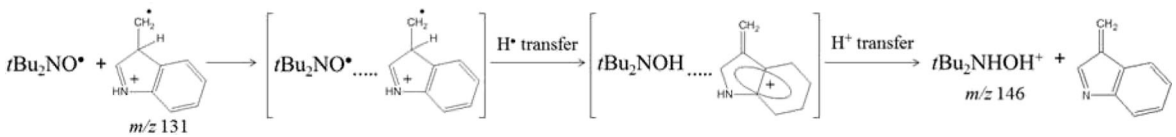
To evaluate individual steps of the theoretical mechanism experimentally, we examined the gas-phase reactivity of the ion *m/z* 131 formed by two ways. First, the *m/z* 131 ion was isolated in the trap after CID of H_3N^+-TrpN$^\bullet$ (*m/z* 204). Independently, 3MI π-radical cation was formed via in-source dissociation of the ternary complex [Cu^{II}(terpy)(3MI)]$^{\bullet 2+}$.

CID of *m/z* 131 ions independent of the way of their production resulted in a single product at *m/z* 130 (see Supplementary Figure S1) corresponding to the loss of one hydrogen atom. This is consistent with the proposed mechanism, but does not shed light on the structure of the *m/z* 131 ion(s) formed via CID of H_3N^+-TrpN$^\bullet$.

Ion-molecule reactions of the *m/z* 131 ion formed via CID of H_3N^+-TrpN$^\bullet$ with di-*tert*-butyl nitroxide gave rise to two products (see Figure 2a) at *m/z* 144 and *m/z* 146. Contrarily, 3MI$^{\bullet+}$ (**1a**) formed directly from the CID of Cu ternary complex of 3MI only underwent an electron transfer under the same conditions yielding the *m/z* 144 ion (Figure 2b). These observations suggest the simultaneous presence of at least two isomeric *m/z* 131 ions in the case of tryptophan N-radical cation fragmentation. One of the *m/z* 131 isomers, 3MI$^{\bullet+}$ (**1a**), reacts via electron transfer to yield *t*Bu$_2$NO$^+$ (Equation 2), similar to the reaction of the π-radical cation of tryptophan in the gas phase [14], and consistent with experiments in solution on electron transfer to 3MI$^{\bullet+}$ [33]. At the same time, the intermediate radical ion **1** (and possibly **1b** and **1c**) reacts by transferring H$^\bullet$ and H$^+$ to *t*Bu$_2$NO• yielding *m/z* 146, *t*Bu$_2$HNOH$^+$ (suggested mechanism using **1** is shown in Equation 3):

$$3MI^{\bullet+} + t\mathrm{Bu_2NO}^\bullet \rightarrow 3MI + t\mathrm{Bu_2NO}^+ \quad (2)$$

While we did not investigate the structure of the resulting ion at *m/z* 146 in detail, a similar product was formed in the reaction of some other ions (e.g., 4-hydroxypyridinium and **2**, the major fragment in CID of π-radical cation of tryptophan [7, 15, 34]. These data are given in Supplementary Figure S3.) The important observation here is that there is a discernable difference in reactivity between the *m/z* 131 ions produced in two ways shown in Scheme 1. This is consistent with the calculated spin densities shown in Figure 1b – 3MI$^{\bullet+}$. Ion **1a** has the spin delocalized over the π-system, whereas **1** and **1b** have highly concentrated spin densities, making them good candidates to be hydrogen atom donors.



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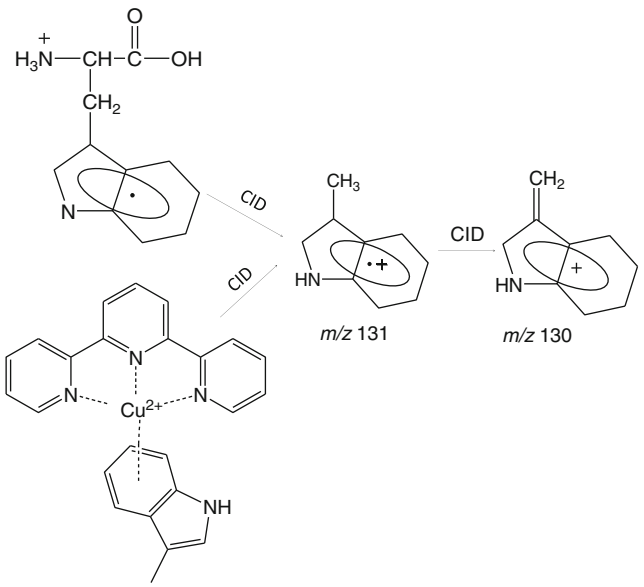


Scheme 1. Possible ways of forming 3-methylindole radical cation: via CID of H_3N^+-TrpN$^\bullet$ obtained from *N*-nitrosotryptophan (top) and oxidation of 3-methylindole via CID of a copper(II) ternary complex (bottom)

H/D Exchange Experiments

We also investigated the fragmentation of the tetradeuterated H_3N^+-TrpN$^\bullet$ at *m/z* 208 (three deuteriums at the N terminus and one at the C terminus, resulting in D_3N^+-TrpN$^\bullet$-COOD) in order to obtain additional confirmation of the proposed mechanism. According to the mechanism shown in Figure 1, two deuteriums (one from the N-terminal D atom transfer to the C3 position, the other from migration of the C-terminal D to the indole N) should be incorporated into the final product, 3MI π-radical cation (**1a**), resulting in an *m/z* 133 fragment. The experimental data shown in Figure 3 clearly show the presence of an ion with *m/z* 134 in the cluster, putting the number of exchangeable hydrogens in the fragment of interest at three (ion *m/z* 135 is present in very low abundance).

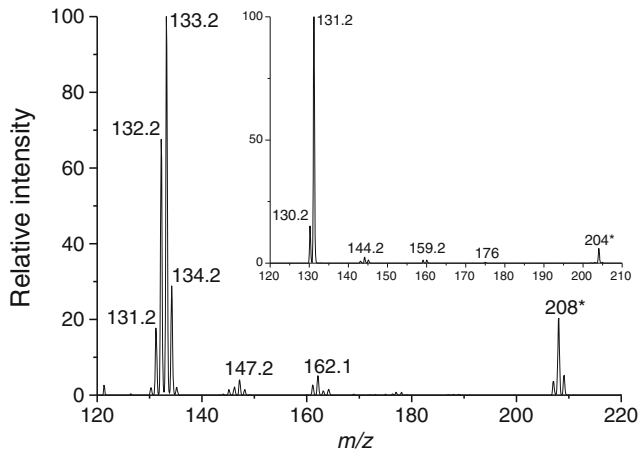
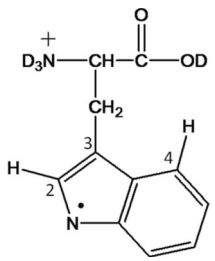


Fig. 3. CID spectrum of deuterated (d_4) tryptophan N-indole radical cation (*m/z* 208). (Inset: CID of tryptophan *N*-indole radical cation, *m/z* 204)

The extra exchangeable hydrogen atom(s) observed experimentally points to a scrambling of one or more aromatic hydrogen atoms in the indole side chain. This explanation is in agreement with earlier works on fragmentation of protonated tryptophan [35], where according to isotopic labeling experiments and theoretical calculations, C2 and C4 positions in the indole side chain were identified as sites of nearly complete scrambling with the protonated N-terminal hydrogens, as shown below.This study also indicated that C_α–C_β bond dissociation is greatly favored over C3–C_β bond cleavage, in agreement with our observations.



Thus, a total of six exchangeable hydrogen atoms (three from the N terminus, one from the C terminus, and two from positions C2 and C4 in the side chain) participate in the formation of the major fragment during CID of H_3N^+-TrpN$^\bullet$. A semiquantitative explanation of the observed experimental intensities of the ions in the *m/z* 131-134 cluster of Figure 3 is given in the Supplement (Supplementary Figure S4 and explanation below).

In conclusion, the fragmentation mechanism of tryptophan *N*-indole radical cation, H_3N^+-TrpN$^\bullet$, under low-energy CID was studied theoretically and experimentally. The main fragment ion, an *m/z* 131 ion, was shown to be a mixture of several isomers, the 3MI π-radical cation (**1a**) and the higher energy *3H*,3–methyleneindolium ion (**1**) and the products of its ring expansion **1b** and **1c**; all of these isomeric ions undergo further loss of a hydrogen atom to yield either the 3-methyleneindolium (**2a**) or quinolinium (**2b**) ions at *m/z* 130. The various stages of the proposed mechanism were verified by CID, H/D exchange, and ion-molecule reactions. 3-Methylindole radical cation (**1a**) was also generated in an independent way and demonstrated gas-phase reactivity similar to a fraction of the *m/z* 131 ions from H_3N^+-TrpN$^\bullet$, thereby providing an extra support for our proposed mechanism.

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