Mining the NIST Mass Spectral Library Reveals the Extent of Sodium Assisted Inductive Cleavage in Collision-Induced Fragmentation.

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ABSTRACT: Interpretation and annotation of fragmentation mass spectra strongly depends on our knowledge of collision-induced fragmentation mechanisms. Computational methods for interpretation of fragmentation operate in the boundaries of recognized fragmentation rules. The prevalence of non-sodiated fragment ions in sodiated ion fragmentation spectra is not yet fully recognized by the mass spectrometry community. Here, we investigated the extent of "Sodium Assisted Inductive Cleavage" (SAIC), a charge migration fragmentation occurring in the fragmentation spectra of sodiated precursors. The NIST17 fragmentation library was mined for evidence of SAIC. A substantial amount of fragment ions in sodiated precursor spectra can be linked to SAIC. Thus, this fragmentation mechanism must be considered to allow for accurate interpretation of fragmentation spectra.

Liquid chromatography coupled with tandem mass spectrometry (LC-MS) is the most common analytical platform used for metabolomics and other small molecule analysis.[1] While initial annotation of LC-MS data can be performed at the MS^1 level (accurate mass, isotope pattern, adduct determination, retention time), more confidence in the annotation can be obtained when supplementing this with fragmentation information from *e.g.* tandem mass spectrometry data collected in non-targeted mode.[2] In this mode, the fragmentation spectra (MS/MS) collected are first searched against spectral libraries, but typically only a few percent can be annotated. The introduction of community-curated spectral libraries such as MassBank[3] and GNPS[4] facilitates the deposition of spectra of identified compounds. Nevertheless, few fragmentation spectra are available for known biomolecules.[5]

Computational methods have been developed that search in molecular structure databases to overcome the limitations of spectral libraries.[6] Combinatorial fragmentation methods such as MetFrag,[7] MAGMa,[8] DEREPLICATOR[9], and MS-Finder[10] attempt to explain fragment peaks with substructures of a query molecule structure. CSI:FingerID[11] uses machine learning to predict a molecular fingerprint for an unknown query spectrum, then uses this fingerprint to search a structure database, but the fingerprint relies on fragment tree annotation made by SIRIUS[12]. All these approaches base their computations on certain chemical assumptions; wrong assumptions may reduce accuracy and performance dramatically. Thus, improving our understanding of fragmentation patterns will help to improve computational methods.

Fragmentation reactions in electrospray ionization (ESI) mass spectrometry are generally classified either as charge retention fragmentation (CRF) or charge migration fragmentation (CMF).[13] Recent studies on diterpene esters using multiple stage mass spectrometry,[14–17] reported the presence of non-sodiated fragment ions in the fragmentation spectra of sodiated precursor ions ($[M+Na]^+$). In ESI, these plant metabolites often form $[M+Na]^+$ adducts and produce rich fragmentation spectra that are dominated by sodiated fragment ions following CRF via a hydrogen rearrangement.[13] The presence of non-sodiated fragment ions in fragmentation spectra of these $[M+Na]^+$ fragmentation spectra raises the question of how frequently this unusual CMF pathway occurs.

In this brief report, a fragmentation mechanism hypothesis for this CMF mechanism in the fragmentation spectra of $[M+Na]^+$ is formulated. We demonstrate that CRF and CMF occur jointly in many fragmentation spectra of $[M+Na]^+$ ions and that this manifests itself by a mass difference of 21.9819 Da, termed "Sodium Assisted Inductive Cleavage" (SAIC). By searching for the SAIC mass difference in the NIST17 spectral library, we describe the frequency of occurrence of this SAIC fragmentation mechanism, and the chemical properties associated with this CMF mechanism.

MATERIALS AND METHODS

Fragmentation pathway of a representative diterpene ester. The fragmentation spectrum of a representative diterpene ester, 12**β**-O-[deca-2Z,4E,6E-trienoyl] 13-isobutyroyloxy 4***β***-deoxyphorbol, was obtained from the GNPS library (CCMSLIB00000840551), and used to annotate its fragmentation pathway. This molecule was isolated from Euphorbia dendroides,[16] and analyzed by non-targeted LC-MS/MS on an LTQ-XL Orbitrap and deposited on MassIVE (MSV000080502).[14] Other diterpene esters with the same fragmentation behavior were characterized by LC-MS/MS[17–19] and can be found on the GNPS library, including phorboids and jatrophane diterpenoids.

Preparation and annotation of the NIST17 MS/MS Spectral Library. The NIST MS/MS 2017 (NIST17) spectral library was mined to explore the extent of the SAIC in sodiated ion CID fragmention.[20,21] The NIST17 library was exported as .MSP format using the *LIB2NIST* converter software (574,826 spectra). In the NIST17, molecular structures are labeled with their InChIKeys and chemical name. In NIST17, spectra are normalized to a base peak intensity of 999. Low resolution spectra were excluded. Fragmentation spectra were combined into a *merged spectrum* if these had the same precursor *m/z*, InChIKey and consecutive NIST IDs; these spectra correspond to different collision energies acquired for the compound on a specific analytical platform. Fragment ions were merged and their intensities added if their mass error was lower than 5 ppm or 0.001 Da. After the merging, only fragment ions above 1 % intensity of the base peak were retained and intensities were normalized to one. Pairs of sodiated and protonated spectra of the same molecular structure were established by matching InChIKey. This resulted in 1803 pairs.

The classification of structures using the ChemOnt chemical ontology was performed by querying ClassyFire[22] with the InChIKeys, resulting in 1,735 annotated spectral pairs. Additionally, SMILES were retrieved for the compounds by searching PubChem with the InChIKeys (25 Nov 2019): a SMILES string was accepted if PubChem contained a single unambiguous SMILES for the compounds' InChIKey plus chemical name, resulting in 1,149 spectral pairs with SMILES annotations. Based on the SMILES annotations, functional groups were counted using ChemmineOB (version 2.30.2)[23] package in R (version 3.4.4).[24]

Analysis of the NIST17 spectral library. Fragment ions in the spectra from NIST17 were attributed to the SAIC or CRF pathway by 1) matching fragment ions between the fragmentation spectra of protonated and sodiated precursor ion or 2) searching for a characteristic *mass difference* in the fragmentation spectrum of the $[M+Na]^+$ ion. A maximum error of 5 ppm and 0.001 Da was considered to match fragment ions or *mass differences*. In the following, the exact *m/z* values are reported. For the fragmentation spectrum of $[M+Na]^+$ ion, it was assumed that a fragment ion originated from the SAIC pathway if it matched another fragment ion in the $[M+H]^+$ spectrum; these are called "*shared fragment ion"* below. The other way around, a fragment ion was assumed to originate from CRF pathway if another fragment ion had a mass difference of 21.9819 Da between the paired spectra, corresponding to the mass of one sodium minus one hydrogen (Na-H). This characteristic Na-H *mass difference* was searched directly within the $[M+Na]^+$ merged spectrum or searched between the merged spectrum of $[M+H]^+$ and $[M+Na]^+$ ions.

RESULTS AND DISCUSSIONS

First, the fragmentation mechanism that leads to non-sodiated fragment ion(s) was examined in more detail. To do so, the fragmentation spectrum of the $[M+Na]^+$ ion formed by a representative diterpene ester was used to interpret the SAIC mechanism occurring under CID (Figure 1). The results showed that the three most intense fragment ions are produced by a CRF mechanism, producing sodiated fragment ions involving intramolecular proton rearrangement(s).

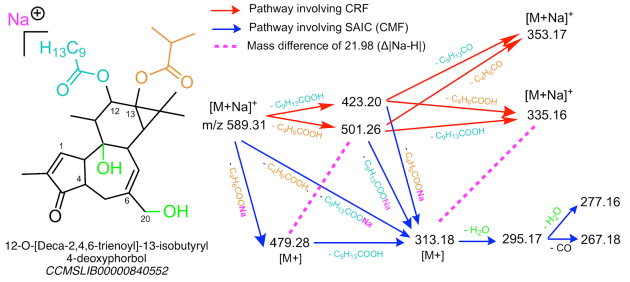

Figure 1. Fragmentation pathways occurring in collision-induced dissociation for the sodiated precursor ion of the 12-O-[deca-2,4,6-trienoyl]-13-isobutyroyloxy 4-deoxyphorbol under a 30V normalized collision energy.

These fragment ions (*m/z* 501.2642, 423.2148, 353.1735, and 335.1596) account for 88.5 % of the total ion count (TIC) in the studied spectrum. The CMF pathway produced low intensity fragment ions [M+]. The four most intense fragment ions produced by SAIC account for only 5.4% of the TIC (*m/z* 479.2771, 313.1791, 295.1686, 277.1593, and 267.1759), which suggests that this CMF pathway is thermodynamically less accessible than the CRF pathway for this sodiated ion. Nevertheless, the $[M^+]$ fragment ions produced by CMF are critical for the determination of the diterpene backbone.[17,25] Our analysis determined it can be established that a mass difference of 21.9819 Da is characteristic of a fragment ion produced by CRF (sodiated) and CMF (non-sodiated), such as *m/z* 501.2641 and 479.2771, or *m/z* 335.1597 and 313.1791. This same mass difference was also observed in the $[M+Na]^+$ spectra of other diterpene esters available on GNPS (Figure S1-S2), supporting the premise that our approach to detecting SAIC is valid.

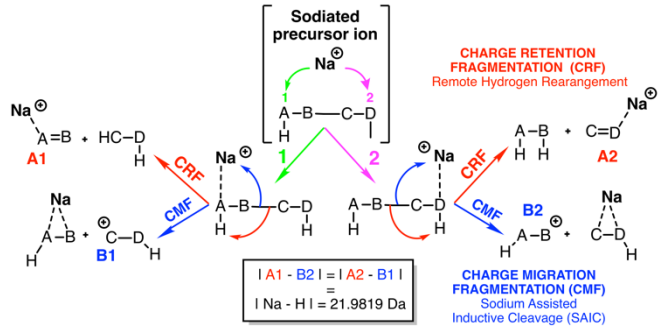

Figure 2. Fragmentation pathways occurring in collision-induced dissociation of sodiated precursor in ESI. (A) Remote hydrogen rearrangement is the most observed mechanism and produces sodiated fragment ions (charge retention fragmentation, CRF). (B) Sodium assisted inductive cleavage (SAIC) is a charge migration fragmentation (CMF) that produces non-sodiated fragment ions. A difference of 21.9819 Da is characteristic of the simultaneous occurrence of the two fragmentation pathways.

Figure 2 describes the simultaneous occurrence of the CRF and CMF pathways in $[M+Na]^+$ fragmentation spectra. The CRF pathway involves an intramolecular proton rearrangement resulting in sodiated fragment ions and the elimination of neutral fragments. This is the best-known fragmentation mechanism for sodiated ions.[13] The SAIC pathway is caused by an intramolecular cleavage involving an intramolecular transfer of electrons from the molecule to the sodium, leading to the production of non-sodiated fragment ion(s), and the neutral loss of the sodium ionically bonded to an anionic molecular fragment. The occurrence of CMF pathway is well recognized in the fragmentation spectra of proton containing ions adducts (*i.e.*

$[M+H_2O+H]^+$ or $[M+NH_3+H]^+$)[25], and in lithium adduct, another alkali metal element.[26,27] The CMF pathway in $[M+Na]^+$ fragmentation spectra has not been formally reported and its extent for fragmentation spectra annotation has never been assessed.[28]

To better understand the prevalence of SAIC in small molecule fragmentation spectra, the NIST17 spectral library was investigated. While fragment ions cannot be identified directly as products of SAIC or CRF, the corresponding fragmentation pathway can be inferred from the observation of mass difference between fragment ions. To do so, the fragmentation spectra of molecules with both sodiated and protonated ions spectral pairs were mined (Figure 3a).

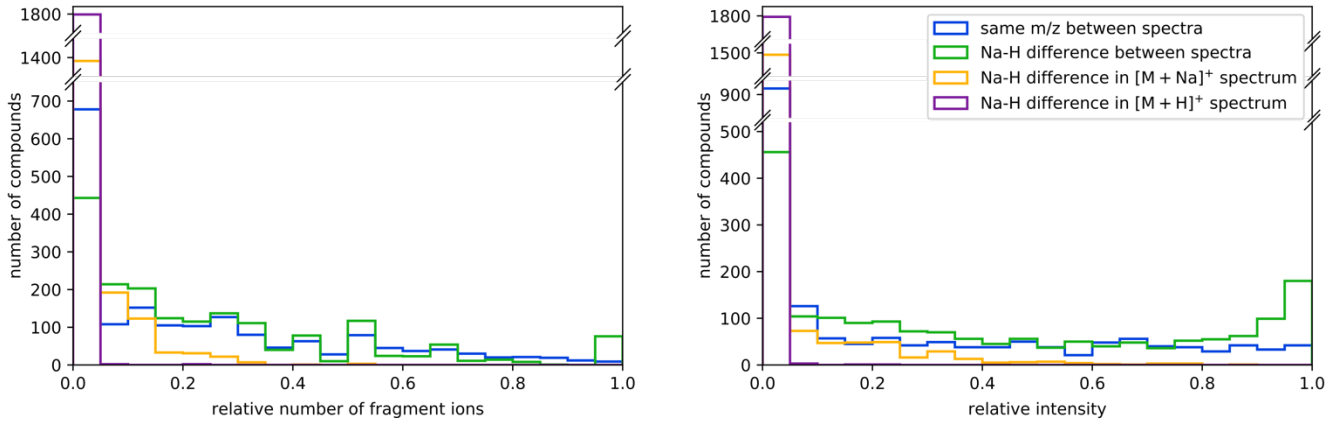

Figure 3. Histogram of (a) the relative number of fragment ions and (b) the summed relative intensity of fragment ions that can be matched between pairs of of $[M+H]^+$ and $[M+Na]^+$ fragmentation spectra: shared fragment ions between spectra (blue) with the same *m/z* value indicate the occurrence of SAIC fragmentation in $[M+Na]^+$ spectra, while the Δ(Na-H) difference between fragment ions for the spectral pair (green) is indicative of sodiated fragment ions. In addition, the frequency of the Δ(Na-H) mass difference in $[M+Na]^+$ spectrum was investigated (gold). The same Δ(Na-H) difference was also searched within the $[M+H]^+$ spectrum (violet). Note the broken y-axis.

Shared fragment ions that are observed in both between $[M+H]^+$ and $[M+Na]^+$ ion fragmentation spectra (0 Da difference) are non-sodiated. In the $[M+Na]^+$ fragmentation spectrum, these *shared* fragments must originate from the SAIC pathway. On the contrary, if the fragmentation spectra of a $[M+H]^+$ and $[M+Na]^+$ spectral pair contain a mass difference of 21.9819 Da - corresponding to the mass of sodium minus hydrogen (Na-H) - it shows sodiated fragment ions originating from the CRF pathway in the $[M+Na]^+$ spectrum. Moreover, the characteristic Na-H difference was searched directly within the sodiated ion spectra to find pairs of [M]+ and $[M+Na]^+$ fragment ions. It could be observed that a large proportion of fragment ions are shared between the $[M+H]^+$ and $[M+Na]^+$ fragmentation spectra: 21.76% of fragment ions in the $[M+Na]^+$ spectra have a corresponding fragment ion in their paired protonated ion spectra. Even more (24.35%) are matched by a Δ(Na-H) difference. Only 0.01% of all fragment ions in $[M+Na]^+$ spectra match ambiguously. In addition, results showed that fragment ions differing by Δ(Na-H) in $[M+Na]^+$ spectra are less frequent showing that SAIC and CRF pathways is not always observed simultaneously in $[M+Na]^+$ spectrum. The relative proportion of annotated fragment ions from the CRF and SAIC pathway is presented in Figure S3 and S4. To exclude that two fragment ions have a Δ(Na-H) mass difference just by chance, the same difference was searched within the protonated ion spectra. This resulted in very few matches showing that fragment ions are rarely matching by chance.

The summed relative intensity of fragment ions for a pathway was examined (Figure 3b). The majority of $[M+Na]^+$ spectra were dominated by sodiated fragment ions, while few contained primarily non-sodiated ions. The distribution of the relative intensities for the individual fragment ions shows that the SAIC pathway in the $[M+Na]^+$ spectra tends to produce lower intensity fragment ions than the CRF pathway (Figure S5).

Next, to investigate if some chemical properties were associated with SAIC, we searched for frequent mass differences between $[M+H]^+$ and $[M+Na]^+$ fragmentation spectra. In total one million mass differences between fragment ions of spectral pairs were randomly sampled. Sampled values were rounded to 3 decimal places and counted to select the most frequent mass differences (Table S1). Results showed the most frequent mass differences were 21.982 Da corresponding to Δ(Na-H), and 0.000 Da (0.91%), along with mass differences indicative of Δ(Na-H) plus loss of water (39.992 Da, 0.51%) and Δ(Na-H) plus a loss of a carbonyl (49.977, 0.23%). The relationship between chemical functions and SAIC was studied by looking at the frequency of functional groups for whether their presence increases the probability of observing a SAIC fragment ions (Figure 4).

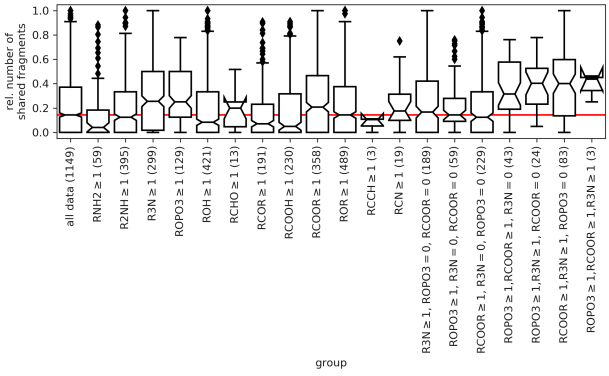

Figure 4. Ratio of shared fragment ions between $[M+H]^+$ and $[M+Na]^+$ of the same compound depending on the existence of various functional groups. Shared fragment ions are used as an approximation for the ratio of non-sodiated fragment ions in $[M+Na]^+$spectra. The boxes indicate the 25th and 75th percentile, the median and its confidence interval. The red line marks the median ratio of shared fragment ions for all available data. For these groups further combinations are displayed. The numbers in brackets indicate the sample size.

The relationship between chemical functions and SAIC was studied by looking at the frequency of functional groups for whether their presence increases the probability of observing a SAIC fragment ions (Figure 4). Results showed that the combination of at least two of the functional groups RCOOR, $ROPO_3$ and R_3N favors non-sodiated fragment ions. Thus, this suggests that two situations could be favoring SAIC: (1) acidic functional groups presumably acting as sodium cation counterions or (2) basic moieties on the charged fragment, which would favor intramolecular cation formation.

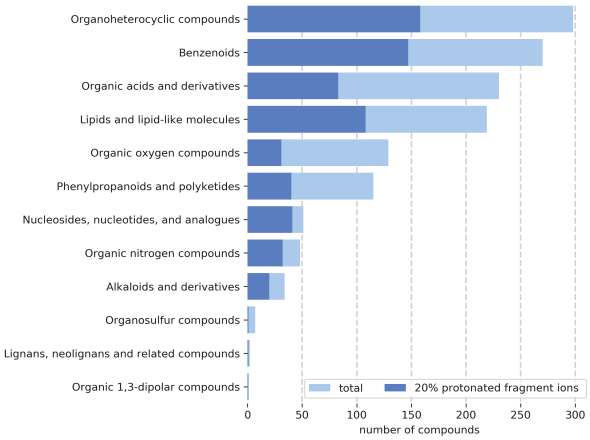

Figure 5. Distribution of the $[M+Na]^+$ spectra in the NIST17 library. Superclass membership of all compounds with greater than 20% relative number of de-sodiated fragments in the fragmentation spectrum of $[M+Na]^+$ spectra is displayed (estimated by searching shared fragments between $[M+H]^+$ and $[M+Na]^+$ spectral pairs). The occurrence of non-sodiated fragment ions in $[M+Na]^+$ spectra is widespread with respect to chemical superclass.

Additionally, it was investigated whether some chemical classes are more prone to SAIC. Therefore, compounds were classified using the ChemOnt chemical ontology.[22] The distribution of the compound superclasses is summarized in Figure 5. This compares the distribution of superclasses for all compounds with ClassyFire annotations (1407) versus compounds with $[M+Na]^+$ spectra enriched with non-sodiated fragments (664 compunds with at least 20% relative number of de-sodiated fragment ions). For robustness, the data was limited to spectra with at least 5 fragment ions. Fragmentation spectra of sodium precursors in the NIST library encompass many ClassyFire superclasses. This diversity suggests that SAIC by CMF is widespread in the $[M+Na]^+$ spectra. Structures of categories "nucleosides, nucleotides, and analogues" and "organic nitrogen compounds" have a high frequency of de-sodiated fragment ions in their sodiated precursor ion fragmentation spectra. This agrees with the observations made above that functional groups $ROPO_3$ and R_3N favor CMF.

CONCLUSIONS

The results support the frequent occurrence of SAIC in the fragmentation spectra of $[M+Na]^+$ across many chemical classes of small molecules. A greater awareness of this phenomenon will improve automated fragment annotation and help avoid missing or incorrect fragment annotations. Thus, methods should account for SAIC and enable the annotation of non-sodiated fragment ions to improve fragmentation spectra annotation of sodiated precursor ion.

ASSOCIATED CONTENT

Supporting Information

A PDF file containing annotated fragmentation spectra, additional statistical analysis conducted in this study, and data from the structures from NIST17 that were analyzed. The Supporting Information is available free of charge on the ACS Publications website.

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Author Contributions

All authors contributed to this work. ML and LFN performed the analysis, with the help of all authors. All authors have approved the final version of the manuscript.

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in Collision Induced Fragmentation