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Epimetabolites: discovering metabolism beyond building and burning

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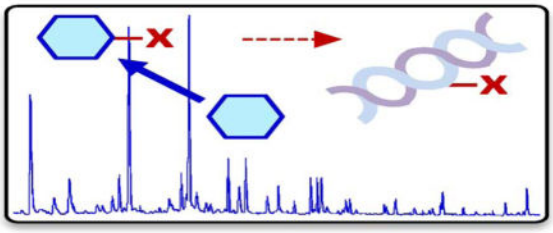
Abstract

Enzymatic transformations of primary, canonical metabolites generate active biomolecules that regulate important cellular and physiological processes. Roles include regulation of histone demethylation in epigenetics, inflammation in tissue injury, insulin sensitivity, cancer cell invasion, stem cell pluripotency status, inhibition of nitric oxide signaling and others. Such modified compounds, defined as epimetabolites, have functions distinct from classic hormones as well as removed from generic anabolism and catabolism. Epimetabolites are discovered by untargeted metabolomics using liquid- or gas chromatography–high resolution mass spectrometry and structurally annotated by *in-silico* fragmentation prediction tools. Their specific biological functions are subsequently investigated by targeted metabolomics methods.

Graphical abstract



Introduction

Metabolism intersects mechanisms in molecular biology with disease endpoints. While new discoveries are still being made in classic central metabolism, the dark matter of the metabolome has been largely ignored [1] (Figure 1). Metabolomics explores the impact of changes in our microbiome [2], repair mechanisms for damaged metabolites [3], the

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exposome [4•] and a range of fields of biologically active metabolites, from oncometabolites [5,6] to the sterol-mediated regulation of the activity of signaling cascades [7,8]. The idea of metabolites with regulatory functions goes well beyond classic biochemical feedback inhibition: metabolites act on distal modules of the molecular and organismal network that are impossible to be explained by the central dogma of unidirectional information flow from genotype to phenotype. This review will focus how targeted and untargeted mass spectrometry methods and software contributes to reveal biological functions of identified and novel, hitherto unknown metabolites.

Beyond building and burning

Metabolomics has focused for too long on classic, well-defined pathways of primary metabolites that constitute the major highways in cellular anabolism or catabolism. This biochemical pathway-centric focus has been nurtured by a view that metabolites only rarely act as regulators, isolating well-researched fields as exceptions to the rule rather than as an overarching theme of metabolism in its own right. Examples include lipid mediators of inflammation [9•], or insulin sensitivity [10••], and regulation of histone demethylases by the oncometabolite 2-hydroxyglutarate (2HG) [11]. These compounds share commonalities that they are often low abundant, transient in nature, and removed from mainstream energy or polymer metabolism. In plants, secondary or specialized metabolites can be considered a synonym to epimetabolites. Specialized metabolites are readily recognized as active regulators and have dedicated and lengthy metabolic pathways for their production [12,13]. Bacteria are also known to use metabolites as regulators for gene expression through riboswitches [14,15]. However, these non-canonical metabolite roles are considered isolated examples and not evidence of a larger role for metabolites *in vivo*.

We are proposing the field of epimetabolites to provide an umbrella term for these non-canonical metabolite functions to fall into. We define an epimetabolite as a metabolite removed from its classical function in anabolism or catabolism. These non-canonical metabolites serve a functional role, including but not limited to, regulation, defense, communication, storage or transport functions. Epimetabolites often remain chemically similar to their canonical counterparts and may use simple modifications like methylation or acetylation that can be easily reversed (Figure 2). They may have once been formed by enzyme errors or chemical damage, but gained biological roles over time. Using untargeted metabolomics, new hypotheses can be generated by discovery of new epimetabolites. Once a new hypothesis is generated, targeted metabolomics methods can be created to accurately quantify them.

Metabolomics methods to target the biological role of epimetabolites

Researchers may be driven by a hypothesis of the involvement of a specific metabolite in biological context: validating such hypothesis is best achieved by targeting this compound by mass spectrometry at high selectivity and sensitivity. To obtain increased specificity, any physicochemical property of small molecules can be exploited to separate metabolites prior to reaching the detector such as differences in boiling point (gas chromatography, GC), lipophilicity (liquid chromatography, LC) electric surfaces charges and migration against a

fluid (capillary electrophoresis, CE) or migration against a gas (ion mobility, IM). Hence, complex mixtures of metabolites can be efficiently separated prior to reaching the mass spectrometer. In addition, sensitivity is enhanced by fragmenting the intact molecules inside the mass spectrometer (MS/MS). By monitoring molecule-specific fragmentations, the signal-to-noise ratio of detection is greatly enhanced because noise molecules or co-eluting compounds that have, by chance, the same mass-to-charge ratio (m/z) and the same chromatographic retention time will unlikely also have the same MS/MS fragmentation. Especially in LC, signal-to-noise ratios are dominated by buffer and solvent clusters, making LC–MS/MS a very effective way to target epimetabolites. To accurately quantify targeted compounds, internal standards and calibration curves are used. Open access software packages for multitarget metabolomics analyses have been reviewed elsewhere [16,17], but lack thorough validation through independent round-robin tests (ring trials).

Commonly measured using targeted metabolomics, oxylipins are prime examples of epimetabolites with very potent regulatory activity. These compounds are released from phospholipid lipases and produced on demand by at least one oxidation step involving molecular oxygen. There are hundreds of different oxylipins in aerobic organisms, ranging from cyclized forms (such as prostaglandins and thromboxanes) to epoxides such as leucotrienes and monohydroxy fatty acids (HETEs). In animals, most oxylipins belong to the family of 20-carbon eicosanoids and have multiple physiological roles, including balancing pro-inflammatory and anti-inflammatory roles in tissue injury. The literature on the roles, regulation and biochemistry of oxylipins abound with hundreds of studies each year. Many metabolomic tools target these classes, with specific emphasis on sample preparation and accurate quantification in plasma by LC–MS/MS [18,19] and on multi-target methods that combine oxylipin profiling with other bioactive lipid classes such as endocannabinoids [20].

A novel class of lipid epimetabolites has recently been discovered, the fatty esters of monohydroxy fatty acids (FAHFAs) [10••]. Specifically, the FAHFA member palmitic acid-9-hydroxystearic acid, 9-PAHSA, was shown to correlate highly with insulin sensitivity and to be reduced in both adipose tissue and serum of insulin-resistant humans. In mice, administering 9-PAHSA improved glucose tolerance while stimulating GLP-1 and insulin secretion, giving mechanistic insights how this new endogenous epimetabolite might act. Once one member of an epimetabolite class has been discovered, detecting similar compounds through metabolomics becomes of high interest. An *in-silico* library of accurate mass MS/MS spectra was generated and experimentally validated, including the detection of previously unknown FAHFA metabolites, to guide the identification of FAHFAs in untargeted metabolomic screens [21].

Targeting methylated epimetabolites

The name “epimetabolite” invokes the analogy to the term epigenetics, a broad class of modifications made outside of changes to gene sequences. Methylation plays and important role in epigenetic regulation and has mounting evidence as an important modification to form epimetabolites. Methylation of classic canonical pathway metabolites such as glycine, nicotinamide or arginine yields epimetabolites with profound cellular or physiological roles.

The oncometabolite *N*-methylglycine, or sarcosine, stimulates invasion and aggressivity in prostate cancer cells, initially discovered through untargeted metabolomics [22]. Further studies show the addition of sarcosine but not glycine or alanine induced tumorgenic changes in *in vivo* prostate cancer models [23]. Another example of a methylated epimetabolite is 1-methylnicotinamde (1MNA), which acts as a methylation sink in naïve embryonic stem cells (ESC) preventing deposit of H2K27me3 marks. Increasing levels of 1MNA, and decreasing levels of S-adenosylmethionine (SAM), are essential to naïve ESC maintenance shown by differentiation of naïve ESC nicotinamide n-methyltransferase knock out line even in the presence of naïve state stabilizers. Targeted and untargeted metabolomics were able to distinguish the primed from naïve in both human and mouse ESCs [24••]. A third example of methylation of canonical metabolites leading to gained regulatory function is asymmetric and symmetric dimethyl arginine (ADMA and SDMA). ADMA and SDMA are produced by the repeated methylation of arginine residues by protein arginine methyltransferases and subsequent proteolysis of methylated proteins. Liberated ADMA, but not SDMA, then works as a competitive inhibitor for endothelial nitric oxide synthase. ADMA inhibition of eNOS can further promote uncoupling of eNOS and production of reactive oxygen and nitrogen species. A meta-analysis of 16 cohort studies involving more than 4,000 subjects showed that ADMA levels alone are significantly associated with an increased risk of coronary artery disease [25]. SDMA itself can be used as biomarker for renal insufficiency, as quantified by targeted LC–MS/MS in urine [26].

Targeting isomeric variants of epimetabolites

When targeting specific metabolites, separating isomers can be critical. Mass spectrometry is usually insufficient in this regard therefore most targeted metabolomic methods utilize chromatographic separation. As biologically relevant compounds are often stereo-specific, it becomes necessary to separate stereoisomers. This can be done with chiral columns and derivatization in LC or GC methods. One example is the well-established oncometabolite 2-hydroxyglutarate (2HG) [27], discovered by untargeted metabolomics [28], which is now known to have different bio-active roles depending upon stereo-conformation. 2HG can be produced by mutated or wild type enzymes in either *R*- or *S*-enantiomer forms respectively (Figure 3). In multiple cancer types, *R*-2HG is produced from mutations to either isocitrate dehydrogenase 1 or 2 (IDH1 or IDH2). Cells without IDH1 or IDH2 mutations can produce *S*-2HG, especially during times of hypoxic stress by either lactate dehydrogenase A or malate dehydrogenase 1 or 2 [29]. Both *R*- and *S*- forms of 2-HG are inhibitors of α-ketoglutarate dependent dioxgenases, which include notably, histone lysine demethylase 4C, an important epigenetic regulator [11]. While both enantiomeric forms of 2HG inhibit cellular demethylases, *S*-2HG is also an inhibitor for EGLN prolyl hydroxylases (involved in HIF-1α regulation) [30••] while *R*-2HG is a substrate for this reaction [29]. Accurate *in vivo* studies of the oncometabolite 2-hydroxyglutarate must therefore include targeted methods capable of enantiomeric separation of *R*- and *S*-2HG.

How to discover and identify new epimetabolites through untargeted metabolomics

As defined above, epimetabolites are chemically modified versions of mainstream compounds that have defined biological roles. While targeted metabolomics methods for these compounds can aid in elucidating their biological roles, it limits the information that can be obtained from samples. However, targeted approaches can be combined with untargeted screening to create a “targeted-plus” method for quantification of metabolites of interest and simultaneous collection of untargeted data [31]. Two steps are involved in discovering genuine metabolites: first, unbiased detection and structural identification of compounds and their statistical association with biological endpoints are collected to yield a starting hypothesis on their potential cellular roles. Second, targeted subsequent studies, as outlined above, give deeper insights into the specifics of their biological functions. Untargeted metabolomics is frequently employed for finding such novel biomarkers, using either GC–MS or LC–MS methods. Typically, multiple extraction and chromatography methods are combined for an untargeted study to capture the greatest diversity of small molecules in a sample. Methods are optimized for coverage and reproducibility. Untargeted metabolomics produces semi-quantitative data on hundreds to thousands of compounds during a single run that are reported by relative intensities. Data processing with classic software such as MZmine 2 [32] and XCMS is still popular, while new subroutines for XCMS have been developed to reduce bias in peak detections [33]. Recently, improved software for peak detection, adduct identification and automated MS/MS deconvolution has been released, MS-DIAL [34••]. MS-DIAL works for both classic data-dependent MS/MS fragmentation experiments as well as data-independent fragmentation studies (Figure 4a). The software also includes large mass spectral libraries such as LipidBlast [35] for compound identifications by MS/MS matching, in addition to scoring deviations from predicted retention times. However, for an overwhelming number of metabolites detected by untargeted metabolomics, no satisfactory MS/MS match can be found.

For the remaining unknown peaks of interest (a combined feature of a specific m/z value at a specific retention time), the hard work starts: the structural annotation of those features. First, the analytical nature of this compounds needs to be defined, for example, as protonated molecular ion $[M+H]^+$ or as one of many other adducts that are regularly detected in LC–MS runs [36]. Only afterwards can one match the accurate mass and isotope distribution information to calculate the most probable elemental composition, the start for annotating the unknown feature by lists of possible isomers. For calculating chemical formulas (Figure 4b), accurate masses are needed with accuracies better than 2 ppm, enabled by instruments that have high mass resolving power (10,000–450,000 FWHM) and relatively wide dynamic linear ranges (3.5–5 orders of magnitude). However, it has previously been shown that accurate mass information alone is insufficient to yield unambiguous elemental formulas, even at <1 ppm mass accuracy [37]. However, if MS/MS fragmentation data are available, the correct elemental formulas are retrieved at the top position in more than 98% of the cases validated for over 5,000 test compounds using the MS-FINDER software [38] or the Sirius 3 algorithm [39].

From elemental formulas to correct epimetabolite structure annotations

Next, all isomer structures of these potential novel epimetabolites need to be searched by their calculated elemental formulas in metabolome databases. A good start is still the Human Metabolome Database (HMDB) [40] but of course, if an epimetabolite indeed has never been reported before, it cannot be retrieved from such resources. Therefore, the scope of possibly existing metabolites has been increased by assuming substrate ambiguity of enzymes, leading to the release of the Metabolic In-silico Network Expansion database (MINE) [41•] that includes more than 571,000 hypothetical compounds, including many metabolites that are derived from simple methylations, acetylations, hydroxylations or other single-reaction modifications that would signify the discovery of an epimetabolite. Albeit, none of these virtual metabolites has a validated MS/MS spectrum, making it difficult to rank the best structure to the experimental MS/MS data. Several research groups have generated tools to predict MS/MS spectra from molecular structures, using chemical bond energies in the improved MetFrag tool [42], known dissociation rules in MS2Analyzer [43], hydrogen bond rearrangements in MS-FINDER [38], fragmentation tree calculations in CSI:FingerID [44] or machine learning strategies in CFM-ID [45•]. These tools are tested in regular competitions, the CASMI contests [46]. At current, the glass is half full, at best as none of these tools yield better than 50% correct structure annotations within the top-5 hits in such blinded tests. There is much room for improvement, and each predicted epimetabolite must still be validated by confirmation using an authentic, synthesized chemical standard. Other approaches include using nuclear magnetic resonance (NMR) which can provide additional insight into unknown structure identification [47].

From identification of potential epimetabolites to defining biological roles

After data processing and identification, known metabolites can be linked to new biological changes, providing new hypotheses to study. For example, the link between microbiome metabolism of phosphatidylcholine and cardiovascular disease was initially discovered using untargeted metabolomics [48]. The epimetabolites discovered as predictors of cardiovascular disease, trimethylamine *N*-oxide (TMAO) and its precursor, γ-butyrobetaine are now appreciated to be important as proatherogenic actors, inducing the development of distinct microbial communities when added to the diet [49]. The recent identification of 4-phosphoerythronate and 2-phospho-L-lactate as side products of mammalian glyceraldehyde 3-phosphate dehydrogenase and pyruvate kinase respectively, illustrate the ability of epimetabolites to regulate classical pathways. 2-Phospho-L-lactate inhibits glycolysis and 4-phosphoerythronate inhibits flux to pentose phosphate pathway. These side products are dephosphorylated by phosphoglycolate phosphatase, now considered to be a metabolite repair enzyme [50]. Other untargeted epimetabolite discoveries may lack specific mechanisms, but have proven to be specific biomarkers for disease states. Diacetylspermine (DAS) has been discovered through untargeted HILIC–QTOF MS/MS as validated marker for non-small-cell lung cancer [51], yet a clear mechanism has yet to be reported. Using untargeted metabolomics, DAS has also been reported to be associated with biofilm formation in colon cancer progression [52]. These examples highlight the difficulties in metabolomics, from untargeted discovery and structure identifications to biological validations.

Conclusions

Metabolites are not mere outputs of genetic networks, but actively participate in many aspects of cellular regulation. The number of regulatory metabolites (beyond classic feedback enzyme inhibition) has expanded to a level that justifies defining a new umbrella classification, epimetabolites. Advances in analytical chemistry have made fast, selective, sensitive and affordable detection of such epimetabolites possible. Hypotheses are generated by finding new epimetabolites through untargeted metabolomics while their biological roles are subsequently validated in targeted metabolomics studies.

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Highlights

- Epimetabolites are modified classic metabolites with new roles
- Example roles include regulation of tumorigenesis, inflammation and pluripotency
- New epimetabolites are discovered by untargeted metabolomics
- Structure annotation by *in-silico* prediction software

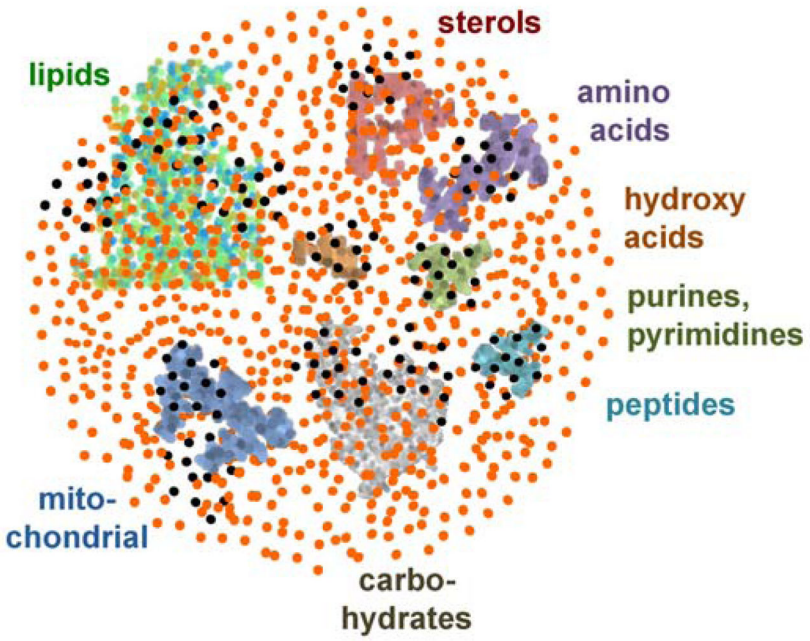

Figure 1.

The dark matter of metabolism. Known biochemical modules (colored) are interspersed with epimetabolites (black), in addition to exposome compounds (orange) detected by untargeted mass spectrometry such as household chemicals, pharmaceuticals, food components, pesticides.

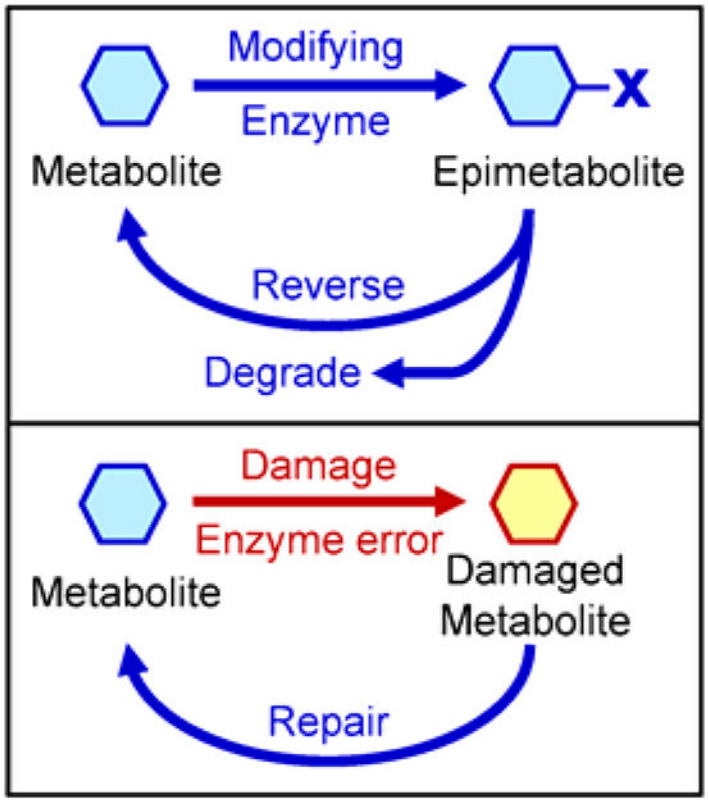

Figure 2.
Origin of novel metabolites. Specific enzymes modify or repair metabolites without creating new pathways.

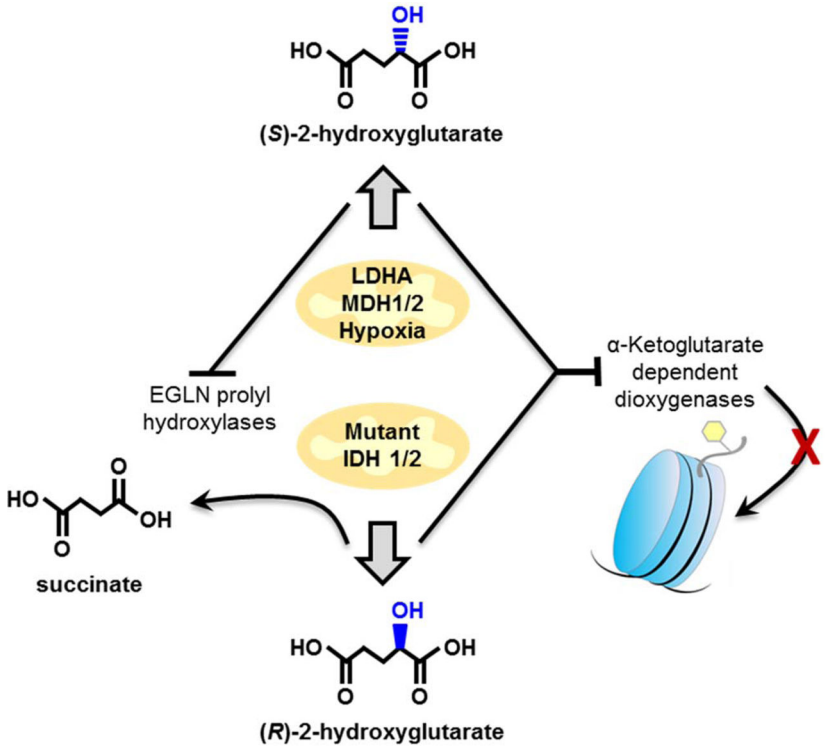

Figure 3.

The different effects of the enantiomeric forms of 2-hydroxyglutarate. Targeted methods must carefully distinguish chiral stereoisomers to unravel the different functions of the oncometabolites (*R*)- and (*S*)-2-hydroxyglutarate that impact histone methylations, or may be substrate to proline hydroxylases.

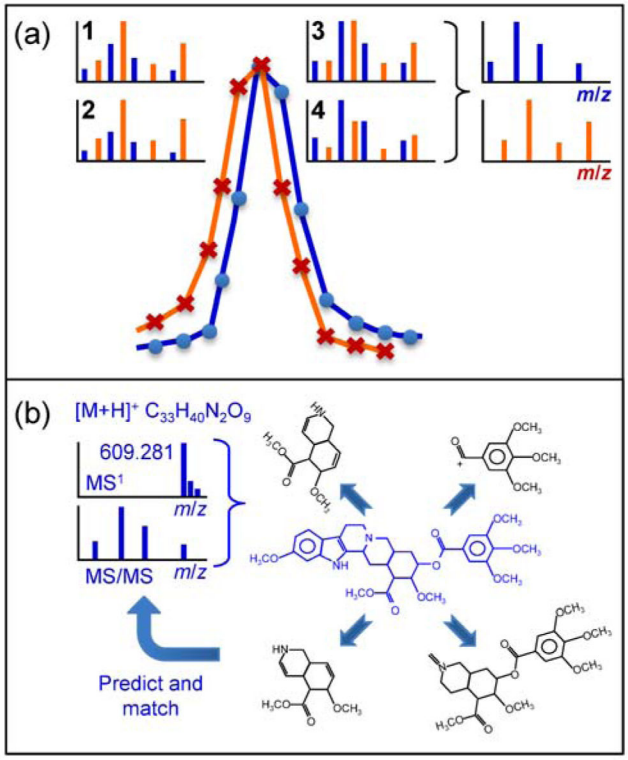

Figure 4.

(a) Mass spectral deconvolution in untargeted metabolomics. In both GC–MS and data-independent LC–MS/MS, molecule fragments overlap for co-eluting metabolites. Following all MS/MS events (1–4) in MS-DIAL enables disentangling precursor and fragment ions to obtain pure MS^1 and MS/MS spectra. (b) Predicting elemental composition and MS fragmentation by *in-silico* tools. From accurate mass, isotope and MS/MS data, elemental formulas are calculated. List of isomer structures are downloaded from databases, MS/MS spectra are predicted, and structures are ranked by highest matching scores.