

Characterising Complex Enzyme Reaction Data

Short title: Characterising Complex Enzyme Reaction Data

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24 **Abstract**

25 The relationship between enzyme-catalysed reactions and the Enzyme Commission (EC) number,
 26 the widely accepted classification scheme used to characterise enzyme activity, is complex and with
 27 the rapid increase in our knowledge of the reactions catalysed by enzymes needs revisiting. We
 28 present a manual and computational analysis to investigate this complexity and found that almost
 29 one-third of all known EC numbers are linked to more than one reaction in the secondary reaction
 30 databases (e.g. KEGG). Although this complexity is often resolved by defining generic, alternative
 31 and partial reactions, we have also found individual EC numbers with more than one reaction
 32 catalysing different types of bond changes. This analysis adds a new dimension to our
 33 understanding of enzyme function and might be useful for the accurate annotation of the function of
 34 enzymes and to study the changes in enzyme function during evolution.

36 **Introduction**

37 Enzymes are life's catalysts that accelerate biochemical reactions up to the rates at which biological
 38 processes take place in living organisms. They play a central role in biology and have been
 39 thoroughly studied over the years. Since the 1960s, the Nomenclature Committee of the
 40 International Union of Biochemistry and Molecular Biology (NC-IUBMB) has systematically
 41 encapsulated the functional information of enzymes into EC numbers. Considered in some cases as
 42 an enzyme nomenclature and classification system, the EC is one way to annotate enzymes, by a
 43 classification of the representative reaction they catalyse, based on multiple aspects of the overall
 44 chemistry such as the chemical bonds that are broken or formed, cofactors being used and the
 45 nature of the substrates undergoing transformation. Introduced into the widely used Gene Ontology
 46 (GO) system for the functional annotation of genes, the EC is the global standard representation of
 47 molecular function for enzymes and relates biological information such as genes, sequence and

48 structure with chemistry data in resources like UniprotKB [1].

49

50 The EC classification as defined by IUBMB is a primary resource for information about enzyme

51 function. Other databases such as KEGG [2] and BRENDA [3] are based around the IUBMB

52 definitions, however in order to handle the flood of data, they associate additional reactions to EC

53 numbers at their discretion, which sometimes causes problems. Nevertheless, the EC has proved to

54 be very powerful. It is manually curated and maintained by expert enzymologists, who use a

55 controlled vocabulary and well-defined relationships in describing enzyme function [4] to convey

56 the way biochemists think about reactions [5]. It facilitates predefined comparisons between

57 enzymes reactions and newly discovered reactions are easily allocated in the different levels of its

58 hierarchical classification. However, because of the diversity of chemical criteria used at different

59 levels, the classification is not coherent between EC classes [6–8]. For instance, lyases (EC 4) are

60 divided in subclasses depending on the type of chemical bond that is broken whereas isomerases

61 (EC 5) are divided based on the type of isomerisation. In addition, the EC classification is based on

62 the overall catalysed reaction, which means that mechanistic steps and reaction intermediates are

63 not considered. As a result, enzymes carrying out the same overall reaction are generally assigned to

64 the same EC number, even when they perform catalysis using different cofactors and mechanisms

65 [9]. For example, three structurally distinct non-homologous chloride peroxidases, which are

66 deemed to have emerged from independent evolutionary events [10,11], catalyse the chlorination of

67 alkanes using three different mechanisms and cofactors. However they are all associated to the same

68 EC number (EC 1.11.1.10). First, vanadate is a prosthetic group in an acid-base mechanism [12]

69 [13]. Second, heme is also a prosthetic group in a radical mechanism [14]. Third, a Ser-His-Asp

70 catalytic triad and an organic acid cofactor are involved in an acid-base mechanism [15]. On the

71 other hand, enzymes catalysing the same overall reaction using the same mechanism with slightly

72 different cofactors are sometimes assigned different EC numbers. For instance, EC 1.1.1.32 and

73 1.1.1.33 represent two mevaldate reductases, both catalyse the conversion of (R)-mevalonate to
74 mevaldate but respectively use NAD^+ and NADP^+ as a cofactor [16].

75
76 Although reliable and rigorous, the manual process of naming each new enzyme and classifying
77 novel enzyme reactions is laborious and requires expert knowledge, therefore automatic approaches
78 may help to accelerate this procedure and to guide the navigation between related enzyme reactions.
79 Similarly, the IUBMB has also considered the current EC classification system to be a relic of the
80 original attempts to develop a chemically sensible hierarchical classification. Ideas and
81 methodologies envisioning a new system in which enzymes are assigned meaningless database
82 identifiers have already been proposed [17] and automatic tools to search and compare enzyme
83 reactions are useful to navigate through “enzyme reaction space” and may help to improve future
84 versions of the classification [18].

85
86 There are biological aspects of enzyme function that are hard to capture in a hierarchical
87 classification system [19]. First, enzymes can be promiscuous and catalyse more than one
88 biochemical reaction [20]. Second, homologous enzymes annotated with the same EC number can
89 manifest different levels of substrate specificity [21] (also known as substrate promiscuity or
90 ambiguity). For instance, UDP-glucose 4-epimerases (EC 5.1.3.2) display different substrate
91 specificities depending on the taxonomic lineage. Bacterial epimerases only act upon UDP-glucose
92 whereas the eukaryotic relatives additionally catalyse the transformation of UDP-N-
93 acetylglucosamine [22]. Even though this limitation has partially been addressed by introducing
94 specificity information in the “Comments” section of several EC entries [23], there is still a need to
95 represent this phenomenon in a more computer-friendly format in order to obtain accurate
96 comparisons between EC numbers. Third, the inclusion of enzyme sequence and structural
97 information would add biological insight to the EC assignment process [21]. This is particularly

98 severe when classifying enzyme functions that involve polymeric biomolecules like sugars, proteins
 99 or DNA. For instance, proteolytic and carbohydrate-active enzymes exhibit broad substrate
 100 specificity and have been alternatively classified using sequence and structure analyses in the
 101 MEROPS [24] and CAZy [25] resources. Fourth, more than 30% of all EC numbers are orphans,
 102 where no enzyme information is known at all [26]. This represents a challenge for the accurate
 103 interpretation of enzyme function in high-throughput sequencing initiatives.

104
 105 Evidence suggests that the correspondences between enzymes, EC numbers and reactions are not
 106 simple [19,27]. The relationship between enzyme and EC number is complex and rarely one-to-one
 107 [10]. Some enzymes are annotated with multiple EC numbers (multifunctional) [5] whereas some
 108 EC numbers are associated with many unrelated enzymes [11]. For example, several studies have
 109 deliberately excluded multifunctional enzymes in order to avoid complexities [28,29]. The
 110 relationship between EC number and reaction is not straightforward either. Although the IUBMB
 111 definitions are the standard, there are striking differences in the way reactions are represented using
 112 the EC classification in several databases. The majority of biologists use the KEGG database in
 113 their work to look at reactions because it provides easy access to chemical equations and molecular
 114 structures for academic users and it is complete in comparison with other databases. Although
 115 various studies exclude reactions associated with more than one EC number [30,31], some
 116 approaches aiming to predict reactivity in metabolites have successfully handled reactions
 117 associated with more than one EC number [32]. To some extent, KEGG circumvents the need for
 118 using EC numbers to link enzymes and biochemical reactions by directly connecting reactions to
 119 groups of orthologous enzymatic genes [33]. This association might considerably simplify the
 120 process of linking chemical and genomic information in the future.

121
 122 This study examines the complexity in the relationship between EC number and reaction in the

KEGG database. Although some reviews mentioned aspects of this connection [26,34], to the authors' best knowledge, studies addressing its complexity in a systematic manner are lacking. We first explored this relationship for a chemically diverse class of enzymes catalysing geometrical and structural rearrangements between isomers, the isomerases. Although this class accounts for only 5.2% of all EC numbers, their diverse chemistry and the similarity of some subclasses to EC primary classes [35], makes the isomerases a class which is representative of the overall chemistry of the EC classification. The knowledge derived from the manual analysis was used to develop an automatic approach to gain an overview of reaction diversity across the EC.

131

132 **Methods**

133 **Overview**

There are 5385 four-digit EC numbers in the 9th April 2014 release of the NC-IUBMB list, 4237 of them (79%) are associated with 6494 unique reactions bearing structural information in the 70.0+ release of KEGG database [2], accessed using the KEGG website and Advanced Programming Interface (API) [36]. The remaining 21% lack structural data. Although most EC numbers are linked to one reaction, almost a third are associated with more than one (Fig. 1a). Comparatively, oxidoreductases (EC 1) exhibit the highest fraction of multiple reactions whereas isomerases (EC 5) the lowest (Fig. 1b). Similarly, some unusual cases were identified where individual EC numbers are linked to over 20 reactions, with one extreme outlier, classified as an unspecific monooxygenase (EC 1.14.14.1) with up to 66 reactions (Fig. 1c). In isomerases, the total number of EC numbers in the database is 245, for which 222 are associated with 298 biochemical reactions and 23 are not linked to any reaction. Among the EC numbers linked to isomerase reactions, 42 are associated with more than one reaction.

146

147 **Fig. 1. Survey of EC numbers associated with more than one enzyme reaction.** (a) Overall

148 distribution. White and grey slices indicate single and multi-reaction EC numbers, respectively. “R-
149 group” represents EC numbers containing a Markush label in at least one reaction (see *Generic*
150 reactions in main text) (b) Distribution by EC class (c) Distribution of EC numbers according to the
151 number of reactions.

152

153 **Automatic analysis – extending diversity groups found in isomerases to the EC** 154 **classification**

155 The automatic extraction of chemical attributes from biochemical reactions such as bond changes is
156 necessary to compare enzymes based on the chemistry of their catalysed reactions. In order to
157 calculate chemical attributes we used EC-BLAST, a recently-developed algorithm to obtain
158 accurate atom-atom mapping, extract bond changes and perform similarity searches between
159 enzyme reactions [18].

160

161 To study reaction diversity across the EC classification, we developed a method based on the 42
162 multi-reaction isomerase EC numbers to automatically label the type of diversity in any multi-
163 reaction EC number (*different* reactants, *generic* reaction on the basis of R-group and
164 stereochemistry, *partial* reaction and *different* types of reactions). The strategy comprised a set of
165 conditional statements combining bond change results from EC-BLAST, which allowed the
166 detection of *different* types of reaction; comparisons of substrate and product structures and
167 identification of R-groups and stereochemistry using Open Babel [37] and in-house scripts, which
168 helped to find *generic* and *partial* reactions (S1 Fig.). Finally, manual analysis of 10% of the
169 remaining multi-reactions EC numbers, which were not detected by the conditions addressing the
170 other diversity groups, revealed them as cases of *different* reactants. This test reduced the bias
171 caused by starting from multi-reaction isomerase EC numbers in the first place.

172

We tested the performance of the method by assessing its ability to correctly identify the type of diversity in fifty randomly-selected multi-reaction EC numbers from the whole of the EC classification. The test dataset comprised 22 oxidoreductases (EC 1), 19 transferases (EC 2), 5 hydrolases (EC 3), 2 lyases (EC 4) and 2 ligases (EC 6), which were manually assigned to a reaction diversity group allowing performance to be evaluated (S2 Fig.). The selection of test multi-reaction EC numbers was carried out randomly, but it was assured that it covers the whole diversity space of the EC classification. Overall, the method successfully assigned the correct diversity group in 41 of the total of 50 test EC numbers. Nine remaining cases could not be correctly assigned due to data errors, detection problems and atom-atom mapping accuracy (S1 Text).

Results

Relationship between EC number and reaction in isomerases

In general, the intrinsic diversity in isomerase multi-reaction EC numbers was interpreted in terms of the chemical variability between the reactions linked to the same EC number. In the context of catalytic promiscuity, previous studies defined reactions to be *different* if they differ in the types of bond changes (formed and cleaved), the reaction mechanism or both [38,39]. The reactions associated with the 42 multi-reaction isomerase EC numbers were manually analysed on the basis of bond and stereochemistry changes and EC numbers were divided into three groups according to *same*, *partial* and *different overall* chemistry of the reaction (Fig. 2). According to our observations, the first group was then further divided into two subgroups: *different* reactants and *generic* reaction. Since the EC number only describes the *overall* reaction, we do not include mechanisms in this analysis. Below is an explanation of each subgroup.

Fig. 2. Examples of isomerase EC numbers associated with more than one enzyme reaction.

(a) Arginine racemase (EC 5.1.1.9) is an isomerase acting on *different* reactants. The variability in

chemical substituents is highlighted in green and the common scaffold in black. (b) Amino acid racemase (EC 5.1.1.10) is an example of *generic* reaction on the basis of R-group. Same colouring as in (a). (c) 2-acetolactate mutase (EC 5.4.99.3) is an example of *generic* reaction based on stereochemistry. The stereochemistry of C2 in acetolactate is represented as straight (undefined), up and down (defined) bonds and highlighted in green. (d) UDP-N-acetyl-D-glucosamine 2-epimerase (EC 5.1.3.14) belongs to *partial* reaction, (i) *overall* reaction – epimerisation of UDP-N-acetyl- α -D-glucosamine (green) and UDP-N-acetyl- α -D-mannosamine (blue), (ii) first *partial* reaction – hydrolysis and epimerisation of UDP-N-acetyl- α -D-glucosamine and (iii) second *partial* reaction – addition of UDP to N-acetyl- α -D-mannosamine. Intermediate compounds are highlighted in red. (e) Dichloromuconate cycloisomerase (EC 5.5.1.11) and 4-chlorobenzoyl-CoA dehalogenase (EC 3.8.1.7) catalyse *different* types of reactions. Shared bond changes are coloured in black, whereas different bond changes in green.

In the *different* reactants subgroup, reaction diversity arises due to the presence of different chemical substituents on a common structural scaffold. For example, the so-called “arginine racemase” (EC 5.1.1.9) describes the racemisation of arginine, lysine and ornithine. The three reactions involve a chiral inversion of the common C α in the amino acid (Fig. 2a).

Generic reactions are used to represent multiple reactions by means of the chemical composition of their reactants. They are represented using Markush labels (e.g. R-groups) [40], which serve as chemical wildcards for other reactions. Almost one in five EC numbers are associated to at least one *generic* reaction, half of them refer to multi-reaction EC numbers and the other half represent single-reaction EC numbers (Fig. 1a). Although the association between Markush labels from the *generic* reaction and the corresponding chemical substructures in exemplar reactions is direct for multi-reaction EC numbers, this correspondence in single-reaction EC numbers is challenging

223 where comparisons with all the other EC numbers are required.

224

225 Multi-reaction EC numbers where at least one reaction is *generic* are the subject of this study. We

226 found that *generic* relationships according to chemical composition are of two types. First, some

227 cases resemble the characteristics of the *different* reactants subgroup but the various chemical

228 substituents are collectively displayed in an additional *generic* reaction, which represents the rest of

229 reactions. For instance, amino acid racemase (EC 5.1.1.10) is linked to five reactions. Four of them

230 describe racemisations of glutamine, serine, ornithine and cysteine and the extra one represents all

231 of them by encapsulating the diversity of the amino acid side chain into a R-group (Fig. 2b). In

232 some cases however, the *generic* reaction is the common structural scaffold shared among all

233 reactions. As a result, there is no R-group involved, and the reactants of the *generic* reaction are

234 substructures of the reactants of the rest of reactions. For example, in Fig. 2a the reactants in the

235 epimerisation of L-ornithine are substructures of the reactants in the epimerisation of L-arginine,

236 hence the former could also be a *generic* reaction of the latter. Although the latter *generic*

237 relationship is evident in our manual analysis, in the process of developing an automatic method to

238 assign EC numbers to reaction diversity groups (see Automatic analysis section) we considered this

239 as an example of *different* reactants. Other isomerase EC numbers fall into this category such as

240 chalcone isomerase (EC 5.5.1.6), which catalyses reversible cyclisation of chalcone into flavanone

241 as common structural scaffold. In addition, it also performs the same reaction in hydroxy-

242 substituted derivatives of chalcone and flavanone [41].

243

244 The second case of representation by *generic* reaction arises due to differences in the definition of

245 stereochemistry between the *generic* reaction and rest of the reactions. Here, undefined

246 stereochemistry (in the form of wiggly or non-stereo bond) characterises one of the chiral carbons

247 in the *generic* reaction, whereas stereochemistry is defined for that atom in the rest of the reactions.

248 Although a previous study reported data challenges due to the lack of stereochemical completeness
 249 in KEGG metabolites and reactions [42], to some extent recent versions of the database have
 250 incorporated these recommendations to improve the handling of stereochemistry and related data
 251 inconsistencies. Taken together, the common existence of cases of defined and undefined
 252 stereochemistry in several EC numbers supported the formulation of this diversity group. For
 253 example, acetolactate mutase (EC 5.4.99.3) is associated with two reactions: the isomerisations of
 254 2-acetolactate (generic reaction, undefined stereochemistry) and (S)-2-acetolactate (specific
 255 reaction, defined stereochemistry) (Fig. 2c). As in *generic* reactions on the basis of R-group, cases
 256 of undefined stereochemistry in the form of wiggly bonds were detected in our automatic method,
 257 however the cases of non-stereo bonds were regarded as examples of *different* reactants.

258
 259 It is a well known fact that there are enzymes releasing intermediate products of an *overall* reaction
 260 from the active site [5]. Reactions leading to these intermediates are known as *partial* reactions.
 261 Similarly, an enzyme may subsequently catalyse two or more *partial* reactions with or without
 262 releasing any intermediates, these are considered as *consecutive* reactions. For example, in Fig. 2d
 263 UDP-N-acetyl-D-glucosamine 2-epimerase (EC 5.1.3.14) catalyses the epimerisation of UDP-N-
 264 acetyl- α -D-glucosamine and UDP-N-acetyl- α -D-mannosamine (*overall* reaction). This
 265 transformation comprises two successive *partial* reactions in the mechanism – hence, they are
 266 *consecutive*. First, the UDP moiety is hydrolytically eliminated from the anomeric carbon and
 267 epimerisation takes place at C2 (first *partial* reaction). Second, the UDP moiety is added to the
 268 anomeric carbon (second *partial* reaction). Combining these two *consecutive* reactions leads to the
 269 *overall* reaction. Whereas this example summarises this group in its simplest form, we also found
 270 three other alternatives of *partial* reactions linked to the same EC number, which are described in
 271 S1 Text. Previous studies have alternatively used the concept of “multi-step reaction” to refer to our
 272 definition of *overall* reaction composed of more than one *partial* reactions that occur consecutively

[6]. However, the term step in a reaction usually implies one mechanistic step of the *overall* reaction. As mechanisms are not included in the EC classification, we preferred using the term *partial* reaction in order to avoid confusion.

Finally, EC numbers might also be linked to at least two *different* types of reactions. Dichloromuconate cycloisomerase (EC 5.5.1.11) catalyses two types: first, the isomerisation of 2,4-dichloro-cis,cis-muconate and 2,4-dichloro-2,5-dihydro-5-oxofuran-2-acetate and also, the conversion of 2,4-dichloro-cis,cis-muconate into trans-2-chlorodienelactone and chloride (Fig. 2e) [43,44]. Although the two reactions share the cleavage of O-H and formation of C-O bonds, they differ in other bond changes, so they are considered to be *different*. However the product of the first isomerisation might eliminate chloride to yield trans-2-chlorodienelactone in an uncatalysed manner and therefore the second reaction would be the result of an isomerisation and successive elimination, which can also be interpreted as an example of *partial* reaction as described before. Other examples of EC numbers that can also be categorised under both *different* types of reaction and *partial* reaction involve sugar isomerisations such as those catalysed by D-arabinose isomerase (EC 5.3.1.3) and ribose-5-phosphate isomerase (EC 5.3.1.6) where the ring opening and closure might be uncatalysed. Perhaps a more definite example of *different* reaction types is 4-chlorobenzoyl-CoA dehalogenase (EC 3.8.1.7). This EC number involves the dehalogenation of 4-chlorobenzoyl-CoA into 4-hydroxybenzoyl-CoA and also the hydrolysis of the fluoro, bromo and iodo derivatives (Fig. 2e). This can also be interpreted as an example of *different* reactants with a halogen atom corresponding to a *generic* substructure.

Following our manual classification, 30 of the 42 multi-reaction isomerase EC numbers were solely assigned to one of the groups, whereas the diversity of the remaining 12 EC numbers was explained by more than one group. Overall, 57 group assignments were manually designated: 24 *different*

reactants, 17 *generic* reactions (R-group and stereochemistry), 5 *partial* reactions and 11 *different* types of reactions. Among the EC numbers assigned to more than one group, we found 2-acetolactate mutase (EC 5.4.99.3) (Fig. 2c). In addition to the transfer of a methyl group from C2 to C3 in (S)-2-acetolactate, this isomerase also catalyses the transfer of an ethyl group from C2 to C3 in (S)-2-aceto-2-hydroxybutanoate. This EC number could be assigned to both groups: *generic* reaction on the basis of stereochemistry and *different* reactants (S3 Fig.). Similarly, although dichloromuconate cycloisomerase (EC 5.5.1.11) is an example of *different* types of reactions (Fig. 2e), a potentially uncatalysed elimination of chloride may also link these two reactions in a *partial* relationship.

307

308 Relationship between EC number and reaction in the EC classification

A schematic diagram illustrating the various groups of reaction diversity is shown in Fig. 3a. There are 1277 multi-reaction EC numbers in the entire EC classification, 90% of them (1153) could be analysed using our method. The most common group was *different* reactants including almost half of the examples. *Different* reaction types followed with 29% and ultimately *partial* and *generic* reactions made up the rest (Fig. 3b). The overall distribution was similar in oxidoreductases (EC 1), transferases (EC 2) and hydrolases (EC 3), which were correspondingly the EC classes involving the highest number of multi-reaction EC numbers (Fig. 3c) and not surprisingly, also the EC classes with the largest number of EC numbers in the EC classification [45]. Exceptionally, the most common diversity group in ligases (EC 6) is *different* reaction types, instead of *different* reactants. Also, the method did not identify any example of EC numbers involving *generic* reactions in lyases (EC 4) and ligases (EC 6).

320

Fig. 3. An overview of reaction diversity in the EC classification. (a) A schematic diagram summarising the groups of reaction diversity. (b) Frequency of reaction diversity group

323 assignments. (c) Total number of multi-reaction EC numbers by EC class for each group of reaction
324 diversity.

325

326 **Discussion**

327 **Overall**

328 Although there is literature reported by the IUBMB discussing specific cases of reaction diversity
329 across the EC classification [5], the aim of this study was to systematically explore aspects of the
330 chemical diversity in the description of enzyme function in a specific EC primary class manually
331 and automatically for the entire EC classification. In order to extract bond changes from reactions
332 we used the EC-BLAST algorithm, which is based on chemical concepts, such as the principle of
333 minimum chemical distance and chemical bond energies, in order to guide the atom-atom mapping
334 and chemical matrices for similarity searches [18]. As suggested in a recent review [46], the
335 incorporation of chemical knowledge adds accuracy to existing strategies to perform reaction
336 comparison.

337

338 This study depends on the quality of reaction data available in the KEGG database [42]. We found
339 this to be the major source of discrepancy between the manual and automatic analyses since many
340 reactions were not balanced hence consistent atom-atom mapping becomes impossible. Whereas
341 multiple strategies to correct unbalanced reactions [46–48] and to reconcile biochemical reactions
342 across databases [34] have been recently presented, novel improvements of the algorithms and
343 further data curation and integration are needed [49,50]. In addition, the quality of the manual
344 curation performed in this study is dependent on the authors' ability to interpret reactions, as well as
345 the experimental information available in the primary literature. The automatic analysis relied only
346 upon the overall reaction equation and the ability of EC-BLAST to compute accurate atom-atom
347 mappings.

348

349 To what extent do the findings of this study overlap with those discovered in previous accounts on
 350 enzyme promiscuity? There are obviously enzymes catalysing different reactions with different EC
 351 numbers, but the IUBMB does not usually include this for most enzymes. However, to some degree,
 352 the working definitions of substrate and product promiscuity [51] somewhat resemble our diversity
 353 groups of *different* reactants and *generic* reactions. Likewise, catalytic promiscuity partly
 354 corresponds to *different* reaction types. However, whereas promiscuity definitions are genuinely
 355 attributed to enzymes in order to describe their ability to catalyse more than one reaction, our
 356 characterisation of reaction diversity applies to diversity within the same EC number, which adds an
 357 extra level of chemical variability to the existing definitions of enzyme function.

358

359 The surprising observation of this study is that almost one-third of the EC numbers involving more
 360 than one reaction have *different* reaction types, bearing key differences in catalysed bond changes.
 361 Whereas some of them also correspond to *partial* reactions, many are cases of catalytic promiscuity
 362 within the same EC number where the annotated enzyme catalyses two or more distinct reactions.
 363 Manual analysis revealed that most cases are similar to 4-chlorobenzoyl-CoA dehalogenase (EC
 364 3.8.1.7) (Fig. 2e) indicating that whereas some bond changes are shared, the rest individually
 365 characterise each of the different reactions.

366

367 The rationale behind why the IUBMB and reaction databases have assigned multiple biochemical
 368 reactions to the same EC number is to some extent comprehensible. For instance, the product of
 369 some catalysed reactions sometimes undergoes a fast and uncatalysed reaction while still in the
 370 active site. These EC numbers comprise two reactions: one comprising only the catalysed reaction
 371 and another consisting of the catalysed+uncatalysed *consecutive* reactions. Whereas some
 372 enzymologists might preferably associate the EC number only with the catalysed reaction, the fact

373 that the uncatalysed reaction takes place in the enzyme's confinement supports the
374 catalysed+uncatalysed interpretation (see Experimental and Results).

375

376 However the complexity in the relationship between reaction and EC number goes beyond this
377 study and cases of *generic* relationships are also common in single-reaction EC numbers (Fig. 1a)
378 and across different EC numbers. For example, as highlighted before, EC 5.1.1.10 was defined by
379 the IUBMB after the discovery of an enzyme that broadly catalyses racemisations of several amino
380 acids [52]. The biochemical reaction contains an R-group and it effectively represents reactions
381 catalysed by specific amino acid racemases, which are also assigned different EC numbers, e.g.
382 alanine (EC 5.1.1.1) and serine (EC 5.1.1.18). Although this and other examples [33] were attempts
383 to incorporate an enzyme property such as substrate specificity to guide the EC classification, this
384 might lead in some cases to EC numbers being embedded into one another and no longer
385 chemically independent from each other, which adds further complications to a classification based
386 solely on the chemistry of the overall reaction.

387

388 **Improving the description of complex enzyme reactions**

389 The ability of the IUBMB to manually update the EC classification in the form of transferred and
390 deleted entries when new enzyme data becomes available is necessary. For example, during the fifty
391 years succeeding the creation of the EC entry for phosphoglycerate mutase in 1961 (EC 5.4.2.1),
392 evidence supporting two distinct mechanisms concerning different usage of the cofactor 2,3-
393 diphosphoglycerate by this enzyme accumulated in the literature [53]. In 2013, the original EC
394 number was transferred to EC 5.4.2.11 (cofactor-dependent) and EC 5.4.2.12 (cofactor-
395 independent). In addition, several expert recommendations concerning definition and handling of
396 EC numbers in biological databases have already been suggested in different contexts. For example,
397 Green and Karp advised about the problems associated with the assignment of partial EC numbers

(those containing a dash, e.g. EC 5.1.1.-) to genes and proposed changes to the specification of these ambiguous identifiers [54]. Similarly, we suggest approaches to clarify multi-reaction EC numbers, which will hopefully help to improve the EC and reaction databases [5] and serve to guide standards for the reporting of enzyme data [55–57] and existing initiatives for the assignment of enzyme function [58–60].

A multi-reaction EC number belonging to the groups' *different* reactants or *generic* reactions could either be combined into a single-reaction EC number (collective approach) or split into as many distinct EC numbers (specific approach). In the first place, diversity could be represented by R-group definitions, which would encapsulate chemical substituents at different positions in the reactants. When necessary, stereochemically-undefined bonds could also be employed to indicate the non-stereoselectivity of some biochemical reactions (Fig. 4a). Secondly, the specific strategy arises when there are significant changes of substrate specificity between enzymes annotated with the same multi-reaction EC number. Instead of defining a *generic* reaction, it might be more sensible to re-define several EC numbers according to the distinct patterns of substrate specificity [61]. However, although EC-BLAST provides a robust method to measure chemical differences between overall reactions in a continuous manner, defining the cut-offs required to designate separate EC numbers (for example, between different substrates) is *a priori* arbitrary and would need to be addressed explicitly.

Fig. 4. Examples of the collective and specific approaches. (a) The *different* reactants of arginine racemase (EC 5.1.1.9) are combined into a single-reaction EC number using R-group. (b) The two *different* types of reaction catalysed by 4-chlorobenzoyl-CoA dehalogenase (EC 3.8.1.7) are split and re-defined into two single-reaction EC numbers.

423 A proposed *modus operandi* when dealing with *different* reaction types involves using the specific
 424 approach to divide the multi-reaction EC number into multiple EC numbers, one for each *different*
 425 reaction [27] (Fig. 4b). Regarding *partial* reactions, we recommend to collectively reduce the multi-
 426 reaction EC number by combining all *partial* reactions with required enzyme catalysis into a single-
 427 reaction EC number, while setting uncatalysed reactions aside.

428

429 Both collective and specific approaches have several benefits. For instance, three main advantages
 430 characterise the collective approach. First, it is a compact way to arrange reaction information in a
 431 clear and structured manner. Second, it conveys how chemists and biochemists represent reactions
 432 in the literature, databases and patents [62–64]. Third, diversity can be captured using Markush
 433 labels such as R-groups [40,65], which would be subsequently described in associated files, tables
 434 or chemical libraries [66]. Alternatively, diversity in the reactants could be encoded using recent
 435 developments in the description of chemical patterns [67]. Also, the collective approach brings
 436 together reactions that are often evolutionarily-related. The precise definition of R-groups will also
 437 help previous studies that were limited in their ability to handle *generic* structures. Although some
 438 strategies did not explicitly define R-groups in their representation of biochemical reactions [68],
 439 several studies preprocessed oxidoreductase (EC 1) and hydrolase (EC 3) reactions by replacing
 440 every R-group by a hydrogen atom [8,69] or methyl group [70] in order to calculate
 441 physicochemical and topological properties in atoms and bonds involved in reaction centres. Using
 442 more specific substitutions, R-groups were manually replaced by methyl, adenine, cytosine or other
 443 chemical moieties depending on the type of biochemical reaction [30,31]. These studies suggest that
 444 having EC number-specific definitions of R-groups based on experimental evidence is a necessary
 445 step in order to implement the collective approach across the classification.

446

447 Whereas the collective approach relies on presenting a common structural scaffold and diversity

448 encoded as chemical placeholders, the specific approach is divisive and explicitly distinguishes
 449 between reactions that are considered as chemically distinct. A clear advantage of the latter is when
 450 subtle differences between biochemical reactions are captured using different EC numbers, for
 451 instance, distinct bond changes or substrate specificity. The description of enzyme function will
 452 then be more detailed and it will help to dissect some of the complexities in the relationship
 453 between enzyme sequence, structure and function [10].

454

455 The terms of the application of the collective and specific approaches to combine or split multi-
 456 reaction EC numbers are proposed in the following recommendations to improve the description of
 457 multi-reaction EC numbers:

458

459 • Reactions sharing the *same overall* chemistry (identical bond changes) should be combined
 460 into a single-reaction EC number (corresponding to groups: *different* reactants and *generic*
 461 reaction). The chemical diversity observed as different embodiments of a *generic* structure
 462 would be encapsulated using R-group definitions and stereochemically-undefined bonds in
 463 associated libraries and chemical patterns.

464

465 • If reactions have *different overall* chemistry (distinct bond changes), the EC number should
 466 be split in multiple single-reaction EC numbers (group: *different* types of reaction).
 467 Similarly, reactions catalysed by enzymes annotated with the same EC number that display
 468 distinct substrate specificities or cofactor dependencies should also be split in as many
 469 single-reaction EC numbers as patterns of specificity exist (groups: *different* reactants and
 470 *generic* reaction).

471

472 • Reactions sharing *partial overall* chemistry (several *partial* reactions integrate into an

overall reaction) should be treated carefully. The *partial* reactions that take place in the active site of the enzyme should be combined into a single-reaction EC number (group: *partial* reaction) with chemical diversity encapsulated in libraries as described before. Uncatalysed *partial* reactions should be considered separately.

As a way to summarise the diversity existing in a multi-reaction EC number, biological databases such as KEGG [2] rely on the so-called “IUBMB reaction”. This is the reaction assigned to the EC number by the IUBMB in the first place, which is chosen by KEGG as the representative reaction for the group of reactions associated with the same EC number (Fig. 4). Whereas this assignment is useful when selecting an example reaction from an EC number and it was adopted as a principle in the development of other reaction databases such as Rhea [71], it is sometimes missing or conflicting and it also overlooks the existing diversity. For instance, EC 5.1.1.13 is described as “Reaction: L-aspartate = D-aspartate” and “Comments: Also acts, at half the rate, on L-alanine”, which is a rather vague description. Similarly, some EC numbers are not associated to any IUBMB reaction and also, EC numbers are sometimes linked to the same IUBMB reaction, 2,3-diphosphoglycerate-dependent and independent phosphoglycerate mutases (EC 5.4.2.11 and EC 5.4.2.12) are both assigned the same IUBMB reaction comprising the isomerisation of 2-phospho-D-glycerate to 3-phospho-D-glycerate. Taken together, from the authors' perspective, a more robust and consistent approach to describe multi-reaction EC numbers is needed.

This systematic analysis is relevant for the functional annotation of sequenced genomes and by extension, it has implications for our ability to build and compare genome-scale metabolic reconstructions [72–74]. There is a direct correspondence between EC numbers and terms representing the molecular function of protein-coding genes in the Gene Ontology (GO) [75], which implicitly adopted EC numbers as part of their classification. This ontology is currently the widely

used standard for the automatic assignment of function to proteins and genes [76]. We observed that multi-reaction EC numbers/GO terms are commonly transferred between similar enzymes during this process. Such a predicted assignment of function does not consider that enzymes annotated with the same multi-reaction EC number might have different reaction specificities in different species, which may lead to a general overestimation of the catalytic capabilities of organisms as predicted from their genomes.

Conclusions

To summarise, this study adds an additional level of chemical complexity to our current description of enzyme function using EC numbers. Remarkably, almost a third of all known EC numbers are associated with more than one enzyme reaction in the KEGG database. Existing approaches to handle this diversity are ineffective, therefore we decomposed this diversity into four categories: *different* reactants, *generic*, *partial* and *different* types of reaction with the aid of computational methods to automatically compare reactions. All multi-reaction EC numbers in our database, annotated according to our reaction typing are given in S1 Table. We hope this information will help to improve our understanding and description of enzyme reactions.

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References

1. The Uniprot Consortium. Update on activities at the Universal Protein Resource (UniProt) in 2013. *Nucleic Acids Res.* 2013;41: D43–7. doi:10.1093/nar/gks1068
2. Kanehisa M, Goto S, Sato Y, Furumichi M, Tanabe M. KEGG for integration and interpretation

- 523 of large-scale molecular data sets. *Nucleic Acids Res.* 2012;40: D109–14.
524 doi:10.1093/nar/gkr988
- 525 3. Schomburg I, Chang A, Placzek S, Söhngen C, Rother M, Lang M, et al. BRENDA in 2013:
526 integrated reactions, kinetic data, enzyme function data, improved disease classification: new
527 options and contents in BRENDA. *Nucleic Acids Res.* 2013;41: D764–72.
528 doi:10.1093/nar/gks1049
- 529 4. Friedberg I. Automated protein function prediction--the genomic challenge. *Brief Bioinform.*
530 2006;7: 225–42. doi:10.1093/bib/bbl004
- 531 5. McDonald A, Tipton K. Fifty--five years of enzyme classification: advances and difficulties.
532 *FEBS J.* 2014;281: 583–592. doi:10.1111/febs.12530
- 533 6. Kotera M, Okuno Y, Hattori M, Goto S, Kanehisa M. Computational Assignment of the EC
534 Numbers for Genomic-Scale Analysis of Enzymatic Reactions. *J Am Chem Soc.* 2004;126:
535 16487–16498. doi:10.1021/ja0466457
- 536 7. Latino DARS, Aires-de-Sousa J. Genome-scale classification of metabolic reactions: a
537 chemoinformatics approach. *Angew Chem Int Ed Engl.* 2006;45: 2066–9.
538 doi:10.1002/anie.200503833
- 539 8. Sacher O, Reitz M, Gasteiger J. Investigations of enzyme-catalyzed reactions based on
540 physicochemical descriptors applied to hydrolases. *J Chem Inf Model.* 2009;49: 1525–34.
541 doi:10.1021/ci800277f
- 542 9. O’Boyle NM, Holliday GL, Almonacid DE, Mitchell JBO. Using reaction mechanism to measure
543 enzyme similarity. *J Mol Biol.* 2007;368: 1484–99. doi:10.1016/j.jmb.2007.02.065
- 544 10. Holliday GL, Fischer JD, Mitchell JBO, Thornton JM. Characterizing the complexity of
545 enzymes on the basis of their mechanisms and structures with a bio-computational analysis.
546 *FEBS J.* 2011;278: 3835–45. doi:10.1111/j.1742-4658.2011.08190.x
- 547 11. Omelchenko M V., Galperin MY, Wolf YI, Koonin E V. Non-homologous isofunctional
548 enzymes: a systematic analysis of alternative solutions in enzyme evolution. *Biol Direct.*
549 2010;5: 31. doi:10.1186/1745-6150-5-31
- 550 12. Messerschmidt A, Wever R. X-ray structure of a vanadium-containing enzyme:
551 chloroperoxidase from the fungus *Curvularia inaequalis*. *Proc Natl Acad Sci U S A.* 1996;93:
552 392–6. Available: <http://www.pnas.org/content/93/1/392.long>
- 553 13. Renirie R, Hemrika W, Piersma SR, Wever R. Cofactor and Substrate Binding to Vanadium
554 Chloroperoxidase Determined by UV–VIS Spectroscopy and Evidence for High Affinity for
555 Pervanadate †. *Biochemistry.* 2000;39: 1133–1141. doi:10.1021/bi9921790
- 556 14. Woggon WD, Wagenknecht HA, Claude C. Synthetic active site analogues of heme-thiolate
557 proteins. Characterization and identification of intermediates of the catalytic cycles of
558 cytochrome P450cam and chloroperoxidase. *J Inorg Biochem.* 2001;83: 289–300.
559 doi:10.1016/S0162-0134(00)00175-6
- 560 15. Hofmann B, Tölzer S, Pelletier I, Altenbuchner J, van Pée KH, Hecht HJ. Structural
561 investigation of the cofactor-free chloroperoxidases. *J Mol Biol.* 1998;279: 889–900.
562 doi:10.1006/jmbi.1998.1802
- 563 16. Shearer AG, Altman T, Rhee CD. Finding sequences for over 270 orphan enzymes. *PLoS One.*
564 2014;9: e97250. doi:10.1371/journal.pone.0097250
- 565 17. Tipton K, Boyce S. History of the enzyme nomenclature system. *Bioinformatics.* 2000;16: 34–
566 40. doi:10.1093/bioinformatics/16.1.34

- 567 18. Rahman SA, Martínez Cuesta S, Furnham N, Holliday GL, Thornton JM. EC-BLAST: a tool to
568 automatically search and compare enzyme reactions. *Nat Methods*. 2014;11: 171–174.
569 doi:10.1038/nmeth.2803
- 570 19. Babbitt PC. Definitions of enzyme function for the structural genomics era. *Curr Opin Chem*
571 *Biol*. 2003;7: 230–7. doi:10.1016/S1367-5931(03)00028-0
- 572 20. O’Brien PJ, Herschlag D. Catalytic promiscuity and the evolution of new enzymatic activities.
573 *Chem Biol*. 1999;6: R91–R105. doi:10.1016/S1074-5521(99)80033-7
- 574 21. Cornish-Bowden A. Current IUBMB recommendations on enzyme nomenclature and kinetics.
575 *Perspect Sci. Elsevier*; 2014;1: 74–87. doi:10.1016/j.pisc.2014.02.006
- 576 22. Daenzer JMI, Sanders RD, Hang D, Fridovich-Keil JL. UDP-galactose 4’-epimerase activities
577 toward UDP-Gal and UDP-GalNAc play different roles in the development of *Drosophila*
578 *melanogaster*. *PLoS Genet*. 2012;8: e1002721. doi:10.1371/journal.pgen.1002721
- 579 23. Kotera M, McDonald AG, Boyce S, Tipton KF. Functional group and substructure searching as
580 a tool in metabolomics. *PLoS One*. 2008;3: e1537. doi:10.1371/journal.pone.0001537
- 581 24. Rawlings ND, Waller M, Barrett AJ, Bateman A. MEROPS: the database of proteolytic
582 enzymes, their substrates and inhibitors. *Nucleic Acids Res*. 2014;42: D503–9.
583 doi:10.1093/nar/gkt953
- 584 25. Lombard V, Golaconda Ramulu H, Drula E, Coutinho PM, Henrissat B. The carbohydrate-
585 active enzymes database (CAZy) in 2013. *Nucleic Acids Res*. 2014;42: D490–5.
586 doi:10.1093/nar/gkt1178
- 587 26. Sorokina M, Stam M, Médigue C, Lespinet O, Vallenet D. Profiling the orphan enzymes. *Biol*
588 *Direct*. 2014;9: 10. doi:10.1186/1745-6150-9-10
- 589 27. Egelhofer V, Schomburg I, Schomburg D. Automatic Assignment of EC Numbers. *PLoS*
590 *Comput Biol*. Public Library of Science; 2010;6: e1000661.
591 doi:10.1371/journal.pcbi.1000661
- 592 28. Des Jardins M, Karp PD, Krummenacker M, Lee TJ, Ouzounis CA. Prediction of enzyme
593 classification from protein sequence without the use of sequence similarity. *Proc Int Conf*
594 *Intell Syst Mol Biol*. 1997;5: 92–9. Available: <http://www.ncbi.nlm.nih.gov/pubmed/9322021>
- 595 29. Todd AE, Orengo CA, Thornton JM. Evolution of function in protein superfamilies, from a
596 structural perspective. *J Mol Biol*. 2001;307: 1113–43. doi:10.1006/jmbi.2001.4513
- 597 30. Latino DARS, Zhang Q-Y, Aires-de-Sousa J. Genome-scale classification of metabolic reactions
598 and assignment of EC numbers with self-organizing maps. *Bioinformatics*. 2008;24: 2236–
599 44. doi:10.1093/bioinformatics/btn405
- 600 31. Latino DARS, Aires-de-Sousa J. Assignment of EC numbers to enzymatic reactions with
601 MOLMAP reaction descriptors and random forests. *J Chem Inf Model*. 2009;49: 1839–46.
602 doi:10.1021/ci900104b
- 603 32. Mu F, Unkefer CJ, Unkefer PJ, Hlavacek WS. Prediction of metabolic reactions based on
604 atomic and molecular properties of small-molecule compounds. *Bioinformatics*. 2011;27:
605 1537–1545. doi:10.1093/bioinformatics/btr177
- 606 33. Kotera M, Goto S, Kanehisa M. Predictive genomic and metabolomic analysis for the
607 standardization of enzyme data. *Perspect Sci. Elsevier*; 2014;1: 24–32.
608 doi:10.1016/j.pisc.2014.02.003
- 609 34. Bernard T, Bridge A, Morgat A, Moretti S, Xenarios I, Pagni M. Reconciliation of metabolites

- 610 and biochemical reactions for metabolic networks. *Brief Bioinform.* 2014;15: 123–35.
611 doi:10.1093/bib/bbs058
- 612 35. Martínez Cuesta S, Furnham N, Rahman SA, Sillitoe I, Thornton JM. The evolution of enzyme
613 function in the isomerases. *Curr Opin Struct Biol.* Elsevier Ltd; 2014;26C: 121–130.
614 doi:10.1016/j.sbi.2014.06.002
- 615 36. Kawashima S, Katayama T, Sato Y, Kanehisa M. KEGG API: A web service using
616 SOAP/WSDL to access the KEGG system. *Genome Informatics.* 2003;14: 673–674.
617 Available: [http://www.jsbi.org/pdfs/journal1/GIW03/GIW03P172.pdf?](http://www.jsbi.org/pdfs/journal1/GIW03/GIW03P172.pdf?origin=publication_detail)
618 [origin=publication_detail](http://www.jsbi.org/pdfs/journal1/GIW03/GIW03P172.pdf?origin=publication_detail)
- 619 37. O’Boyle NM, Banck M, James CA, Morley C, Vandermeersch T, Hutchison GR. Open Babel:
620 An open chemical toolbox. *J Cheminform.* Chemistry Central Ltd; 2011;3: 33.
621 doi:10.1186/1758-2946-3-33
- 622 38. Kazlauskas RJ. Enhancing catalytic promiscuity for biocatalysis. *Curr Opin Chem Biol.* 2005;9:
623 195–201. doi:10.1016/j.cbpa.2005.02.008
- 624 39. Kaltenbach M, Tokuriki N. Dynamics and constraints of enzyme evolution. *J Exp Zool B Mol*
625 *Dev Evol.* 2014;322: 468–87. doi:10.1002/jez.b.22562
- 626 40. Brecher J. Graphical representation standards for chemical structure diagrams (IUPAC
627 Recommendations 2008). *Pure Appl Chem.* 2008;80: 277–410.
628 doi:10.1351/pac200880020277
- 629 41. Kimura Y, Aoki T, Ayabe S. Chalcone isomerase isozymes with different substrate specificities
630 towards 6’-hydroxy- and 6'-deoxychalcones in cultured cells of *Glycyrrhiza echinata*, a
631 leguminous plant producing 5-deoxyflavonoids. *Plant Cell Physiol.* 2001;42: 1169–73.
632 doi:10.1093/pcp/pce130
- 633 42. Ott MA, Vriend G. Correcting ligands, metabolites, and pathways. *BMC Bioinformatics.*
634 2006;7: 517. doi:10.1186/1471-2105-7-517
- 635 43. Kuhm AE, Schlömann M, Knackmuss HJ, Pieper DH. Purification and characterization of
636 dichloromuconate cycloisomerase from *Alcaligenes eutrophus* JMP 134. *Biochem J.*
637 1990;266: 877–83. Available: <http://www.ncbi.nlm.nih.gov/pubmed/?term=2327971>
- 638 44. Pieper D, Stadler-Fritzsche K. Metabolism of 2-chloro-4-methylphenoxyacetate by *Alcaligenes*
639 *eutrophus* JMP 134. *Arch Microbiol.* 1993;160: 169–178. doi:10.1007/BF00249121
- 640 45. McDonald AG, Boyce S, Tipton KF. ExplorEnz: the primary source of the IUBMB enzyme list.
641 *Nucleic Acids Res.* 2009;37: D593–7. doi:10.1093/nar/gkn582
- 642 46. Chen WL, Chen DZ, Taylor KT. Automatic reaction mapping and reaction center detection.
643 *Wiley Interdiscip Rev Comput Mol Sci.* 2013;3: 560–593. doi:10.1002/wcms.1140
- 644 47. Kraut H, Eiblmaier J, Grethe G, Löw P, Matuszczyk H, Saller H. Algorithm for reaction
645 classification. *J Chem Inf Model.* 2013;53: 2884–95. doi:10.1021/ci400442f
- 646 48. Shaw R, Debsarma S, Kundu S. An algorithm for removing stoichiometric discrepancies in
647 biochemical reaction databases. *Curr Sci.* 2012;103: 1328–1334. Available:
648 <http://www.currentscience.ac.in/Volumes/103/11/1328.pdf>
- 649 49. Kumar A, Suthers PF, Maranas CD. MetRxn: a knowledgebase of metabolites and reactions
650 spanning metabolic models and databases. *BMC Bioinformatics.* BioMed Central Ltd;
651 2012;13: 6. doi:10.1186/1471-2105-13-6
- 652 50. Lang M, Stelzer M, Schomburg D. BKM-react, an integrated biochemical reaction database.

- 653 BMC Biochem. BioMed Central Ltd; 2011;12: 42. doi:10.1186/1471-2091-12-42
- 654 51. Hult K, Berglund P. Enzyme promiscuity: mechanism and applications. Trends Biotechnol.
655 2007;25: 231–8. doi:10.1016/j.tibtech.2007.03.002
- 656 52. Lim YH, Yokoigawa K, Esaki N, Soda K. A new amino acid racemase with threonine alpha-
657 epimerase activity from *Pseudomonas putida*: purification and characterization. J Bacteriol.
658 1993;175: 4213–7. Available: <http://www.ncbi.nlm.nih.gov/pubmed/8320235>
- 659 53. Foster JM, Davis PJ, Raverdy S, Sibley MH, Raleigh EA, Kumar S, et al. Evolution of bacterial
660 phosphoglycerate mutases: non-homologous isofunctional enzymes undergoing gene losses,
661 gains and lateral transfers. PLoS One. 2010;5: e13576. doi:10.1371/journal.pone.0013576
- 662 54. Green ML, Karp PD. Genome annotation errors in pathway databases due to semantic
663 ambiguity in partial EC numbers. Nucleic Acids Res. 2005;33: 4035–9.
664 doi:10.1093/nar/gki711
- 665 55. Apweiler R, Armstrong R, Bairoch A, Cornish-Bowden A, Halling PJ, Hofmeyr J-HS, et al. A
666 large-scale protein-function database. Nat Chem Biol. 2010;6: 785.
667 doi:10.1038/nchembio.460
- 668 56. Tipton KF, Armstrong RN, Bakker BM, Bairoch A, Cornish-Bowden A, Halling PJ, et al.
669 Standards for Reporting Enzyme Data: The STRENDA Consortium: What it aims to do and
670 why it should be helpful. Perspect Sci. 2014;1: 131–137. doi:10.1016/j.pisc.2014.02.012
- 671 57. Gardossi L, Poulsen PB, Ballesteros A, Hult K, Svedas VK, Vasić-Racki D, et al. Guidelines for
672 reporting of biocatalytic reactions. Trends Biotechnol. 2010;28: 171–80.
673 doi:10.1016/j.tibtech.2010.01.001
- 674 58. Gerlt JA, Allen KN, Almo SC, Armstrong RN, Babbitt PC, Cronan JE, et al. The enzyme
675 function initiative. Biochemistry. 2011;50: 9950–62. doi:10.1021/bi201312u
- 676 59. Bastard K, Smith AAT, Vergne-Vaxelaire C, Perret A, Zapparucha A, De Melo-Minardi R, et al.
677 Revealing the hidden functional diversity of an enzyme family. Nat Chem Biol. 2014;10: 42–
678 9. doi:10.1038/nchembio.1387
- 679 60. Anton BP, Chang Y-C, Brown P, Choi H-P, Faller LL, Guleria J, et al. The COMBREX Project:
680 Design, Methodology, and Initial Results. PLoS Biol. 2013;11: e1001638.
681 doi:10.1371/journal.pbio.1001638
- 682 61. Schomburg I, Chang A, Schomburg D. Standardization in enzymology -- Data integration in the
683 world's enzyme information system BRENDA. Perspect Sci. Elsevier; 2014;1: 15–23.
684 doi:10.1016/j.pisc.2014.02.002
- 685 62. Warr WA. Representation of chemical structures. Wiley Interdiscip Rev Comput Mol Sci.
686 2011;1: 557–579. doi:10.1002/wcms.36
- 687 63. Zass E. A user's view of chemical reaction information sources. J Chem Inf Model. 1990;30:
688 360–372. doi:10.1021/ci00068a004
- 689 64. Geyer P. Markush structure searching by information professionals in the chemical industry -
690 Our views and expectations. World Pat Inf. Elsevier Ltd; 2013;35: 178–182.
691 doi:10.1016/j.wpi.2013.05.002
- 692 65. Simmons ES. The grammar of Markush structure searching: vocabulary vs. syntax. J Chem Inf
693 Model. 1991;31: 45–53. doi:10.1021/ci00001a007
- 694 66. Warr WA. A Short Review of Chemical Reaction Database Systems, Computer-Aided Synthesis
695 Design, Reaction Prediction and Synthetic Feasibility. Mol Inform. 2014;33: 469–476.

- doi:10.1002/minf.201400052
67. Schomburg KT, Wetzer L, Rarey M. Interactive design of generic chemical patterns. *Drug Discov Today*. Elsevier Ltd; 2013;18: 651–8. doi:10.1016/j.drudis.2013.02.001
68. Triviño JC, Pazos F. Quantitative global studies of reactomes and metabolomes using a vectorial representation of reactions and chemical compounds. *BMC Syst Biol*. 2010;4: 46. doi:10.1186/1752-0509-4-46
69. Hu X, Yan A, Tan T, Sacher O, Gasteiger J. Similarity perception of reactions catalyzed by oxidoreductases and hydrolases using different classification methods. *J Chem Inf Model*. 2010;50: 1089–100. doi:10.1021/ci9004833
70. Mu F, Unkefer PJ, Unkefer CJ, Hlavacek WS. Prediction of oxidoreductase-catalyzed reactions based on atomic properties of metabolites. *Bioinformatics*. 2006;22: 3082–8. doi:10.1093/bioinformatics/btl535
71. Alcántara R, Axelsen KB, Morgat A, Belda E, Coudert E, Bridge A, et al. Rhea--a manually curated resource of biochemical reactions. *Nucleic Acids Res*. 2012;40: D754–60. doi:10.1093/nar/gkr1126
72. Saha R, Chowdhury A, Maranas CD. Recent advances in the reconstruction of metabolic models and integration of omics data. *Curr Opin Biotechnol*. Elsevier Ltd; 2014;29C: 39–45. doi:10.1016/j.copbio.2014.02.011
73. Oberhardt MA, Puchałka J, Martins dos Santos VAP, Papin JA. Reconciliation of genome-scale metabolic reconstructions for comparative systems analysis. *PLoS Comput Biol*. 2011;7: e1001116. doi:10.1371/journal.pcbi.1001116
74. Monk J, Nogales J, Palsson BO. Optimizing genome-scale network reconstructions. *Nat Biotechnol*. 2014;32: 447–52. doi:10.1038/nbt.2870
75. Ashburner M, Ball CA, Blake JA, Botstein D, Butler H, Cherry JM, et al. Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. *Nat Genet*. 2000;25: 25–9. doi:10.1038/75556
76. Radivojac P, Clark WT, Oron TR, Schnoes AM, Wittkop T, Sokolov A, et al. A large-scale evaluation of computational protein function prediction. *Nat Methods*. 2013;10: 221–7. doi:10.1038/nmeth.2340

Supporting Information

S1 Text. Extension of the methods and results described in the manuscript.

S1 Fig. Workflow illustrating the automatic analysis of multi-reaction EC numbers.

S2 Fig. Results of the test to evaluate the automatic method labelling multi-reaction EC

733 **numbers according to the reaction diversity group.**

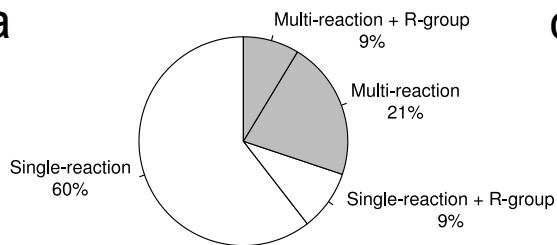
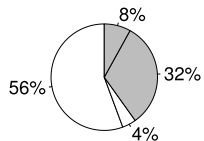
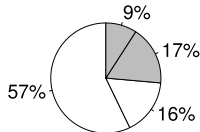
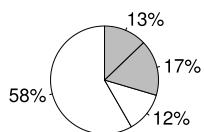
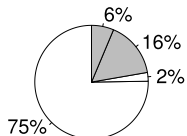
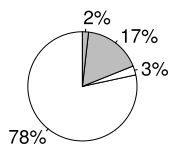
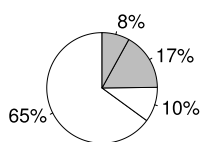
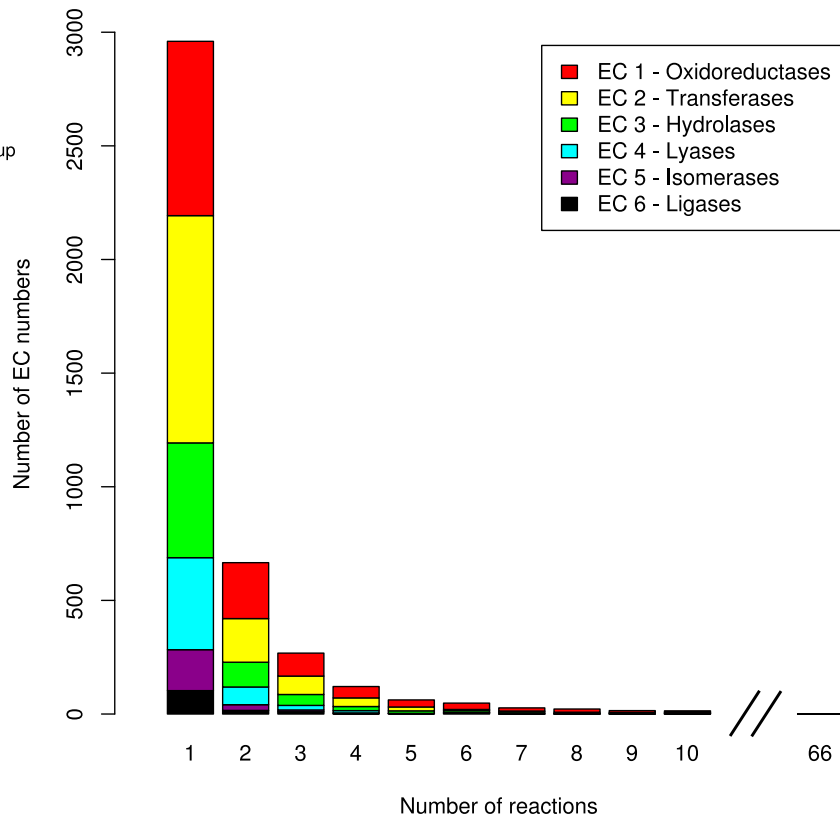
734

735 **S3 Fig. 2-Acetolactate mutase (EC 5.4.99.3) is an example of EC number assigned to two**
 736 **groups of reaction diversity: *different* types of reaction and *partial* reactions.**

737

738 **S1 Table. Table listing all the multi-reaction EC numbers considered in this study.** They have
 739 been annotated according to our description of chemical diversity groups and isomerase EC
 740 numbers have been manually labelled with our recommendation for improvement.

741

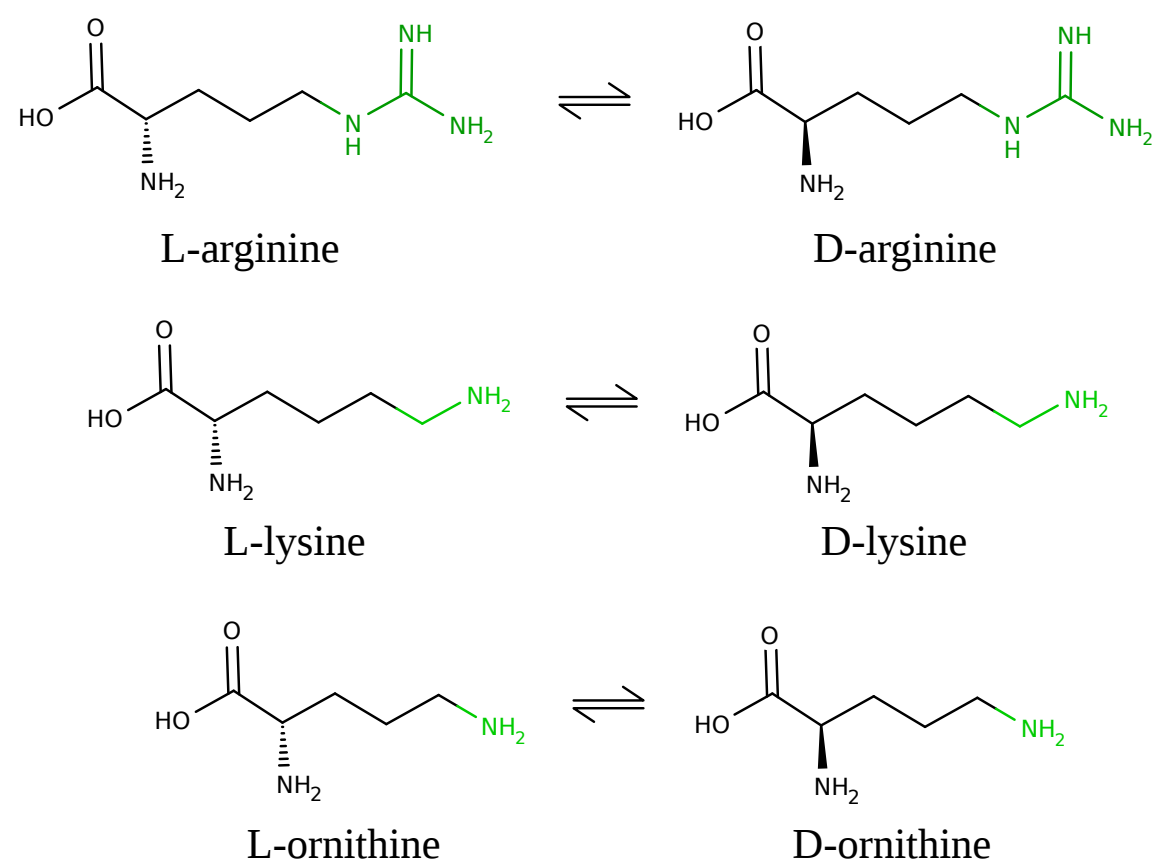
a**b****EC 1 - Oxidoreductases****EC 2 - Transferases****EC 3 - Hydrolases****EC 4 - Lyases****EC 5 - Isomerases****EC 6 - Ligases****c**

Same chemistry

a

Different reactants

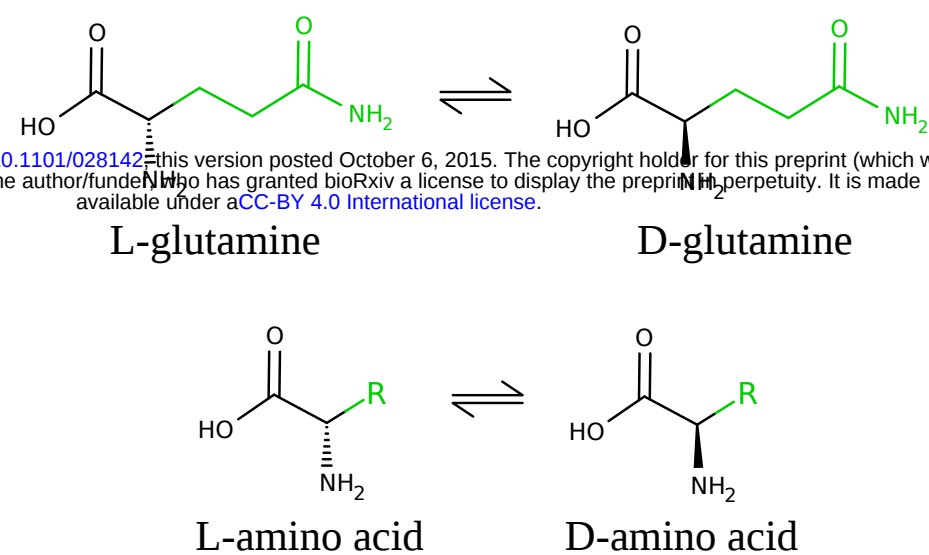
Arginine racemase (EC 5.1.1.9)



b

Generic reaction + R-group

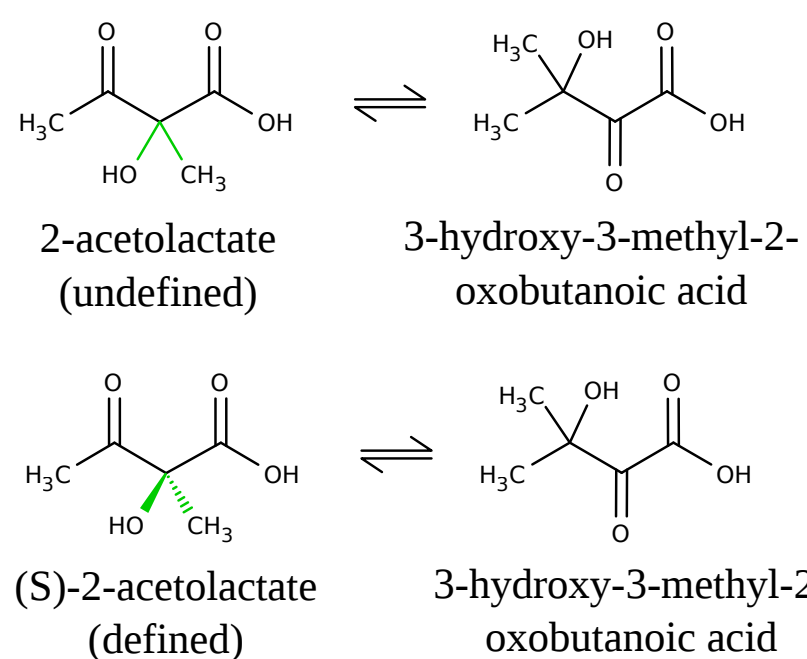
Amino acid racemase (EC 5.1.1.10)



c

Generic reaction + stereochemistry

2-Acetolactate mutase (EC 5.4.99.3)



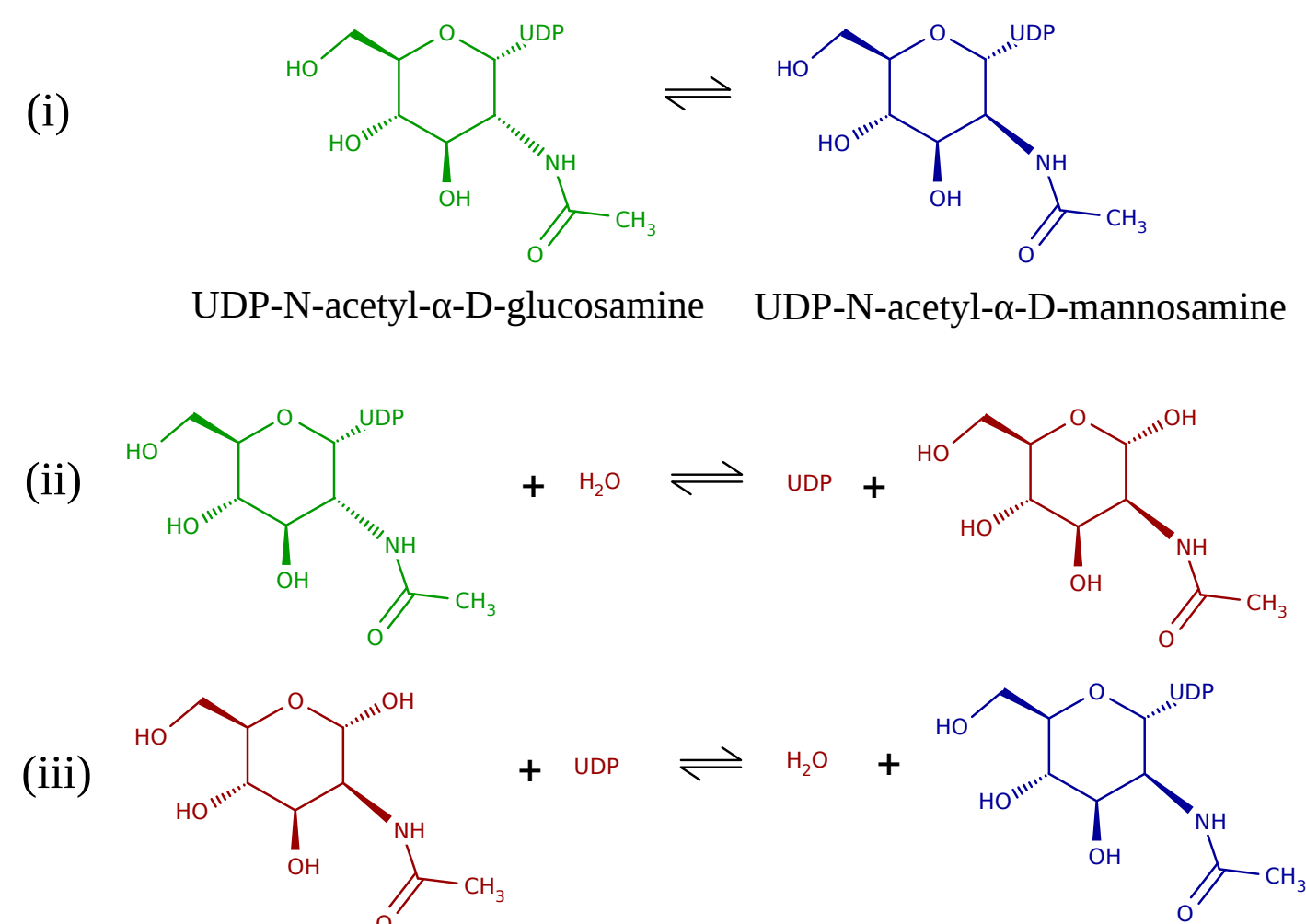
Partial chemistry

d

Partial reaction

UDP-N-acetyl-D-glucosamine 2-epimerase (EC 5.1.3.14)

(i) = (ii) + (iii)

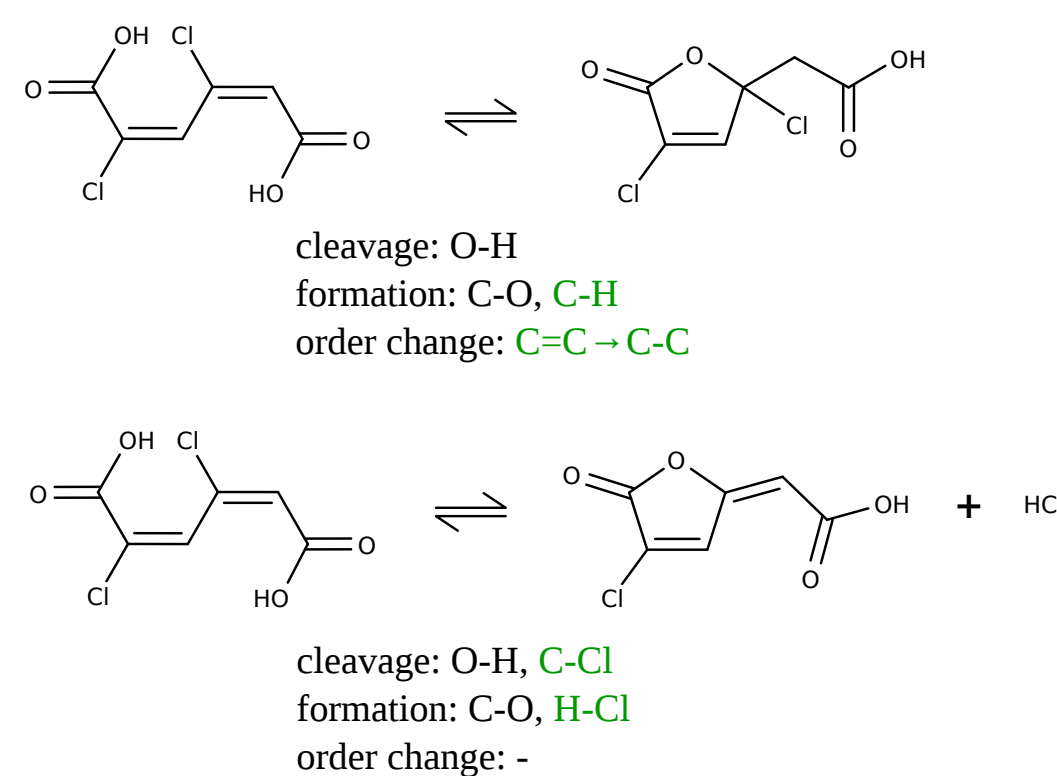


Different chemistry

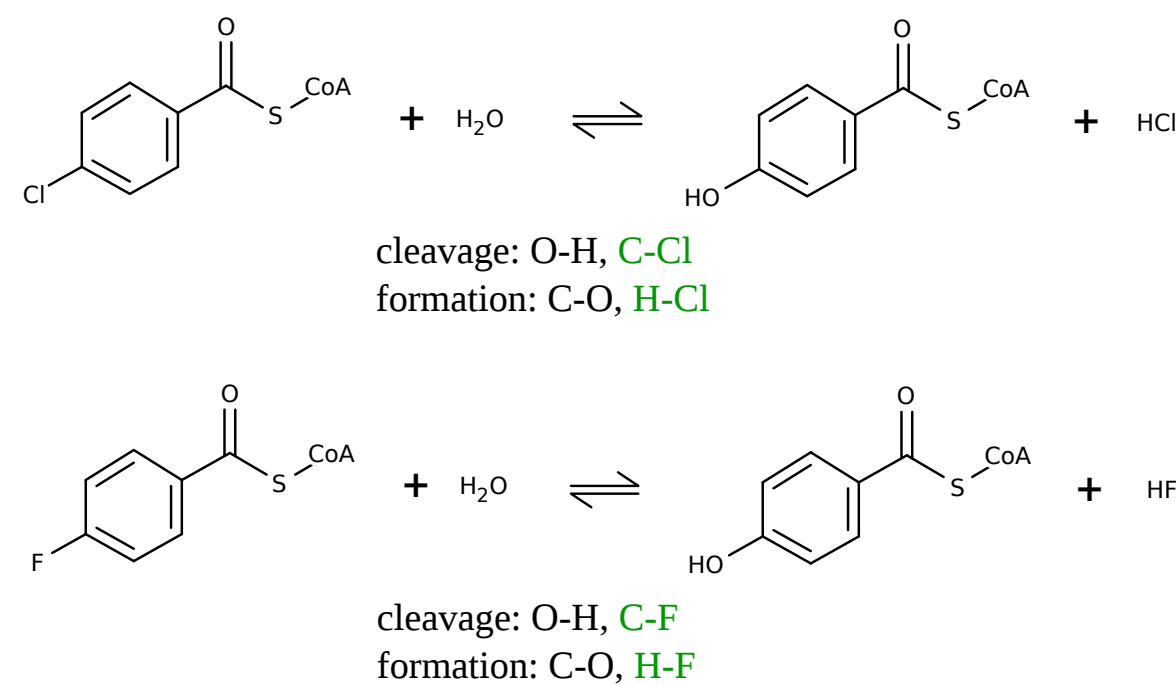
e

Different types of reaction

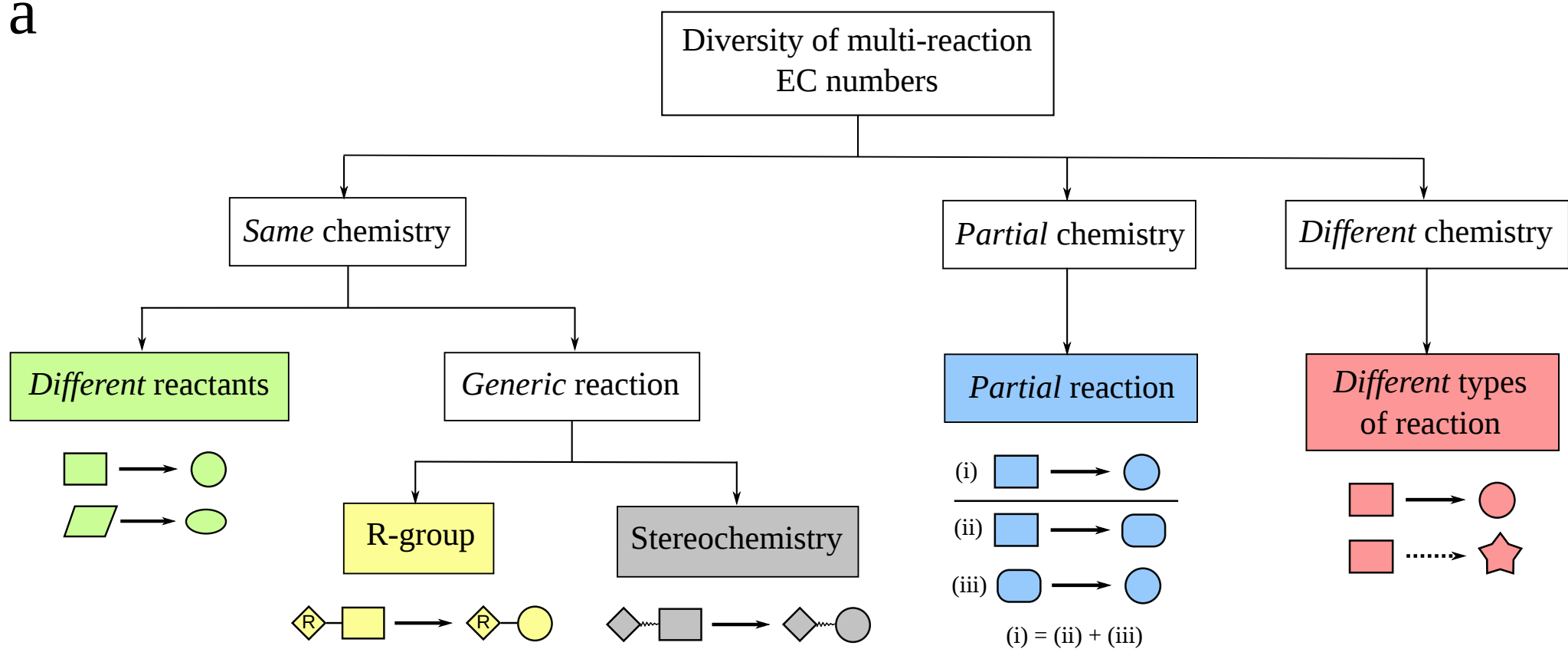
Dichloromuconate cycloisomerase (EC 5.5.1.11)



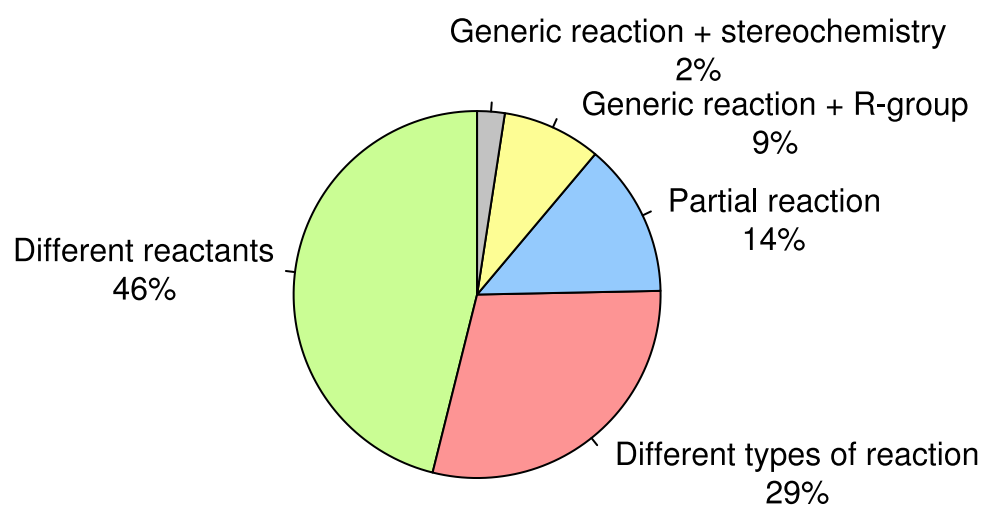
4-chlorobenzoyl-CoA dehalogenase (EC 3.8.1.7)



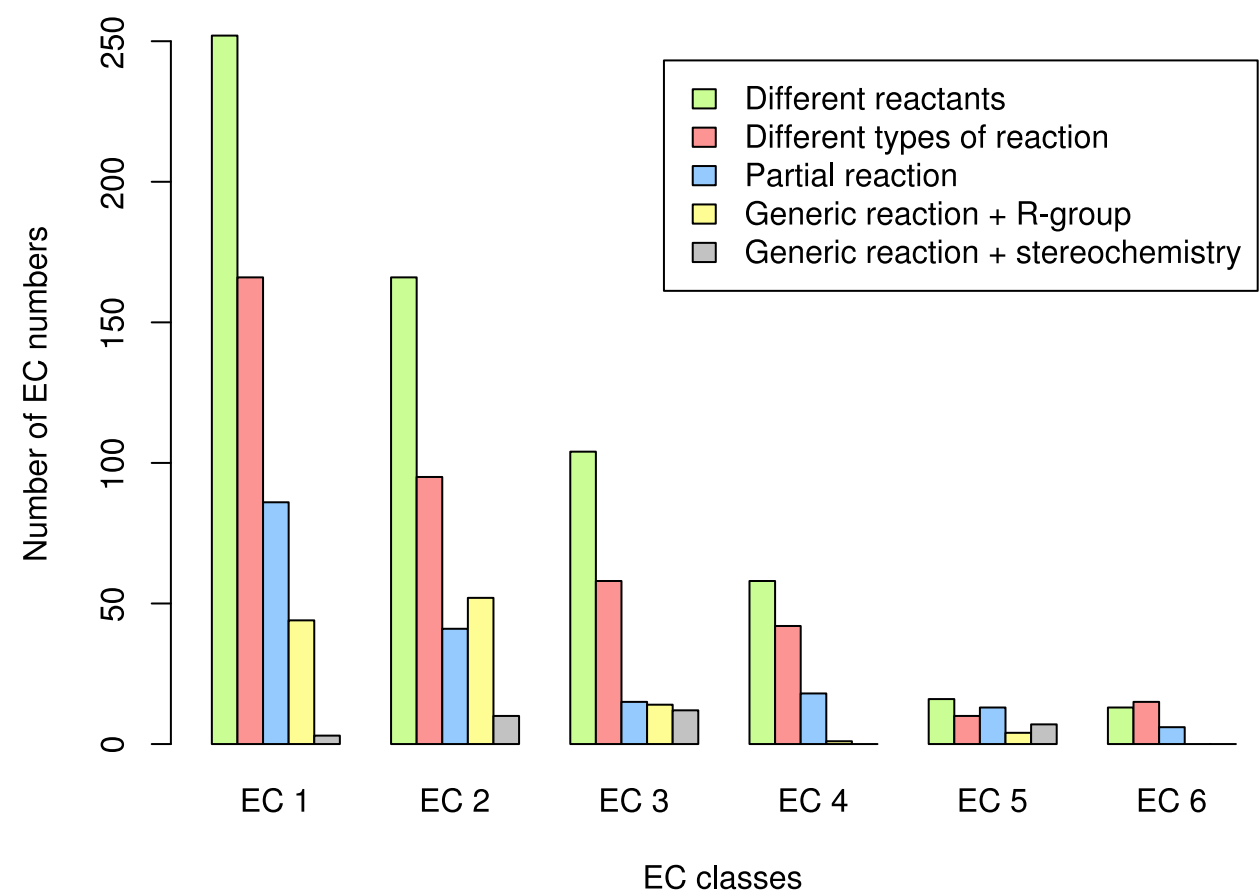
a



b



c

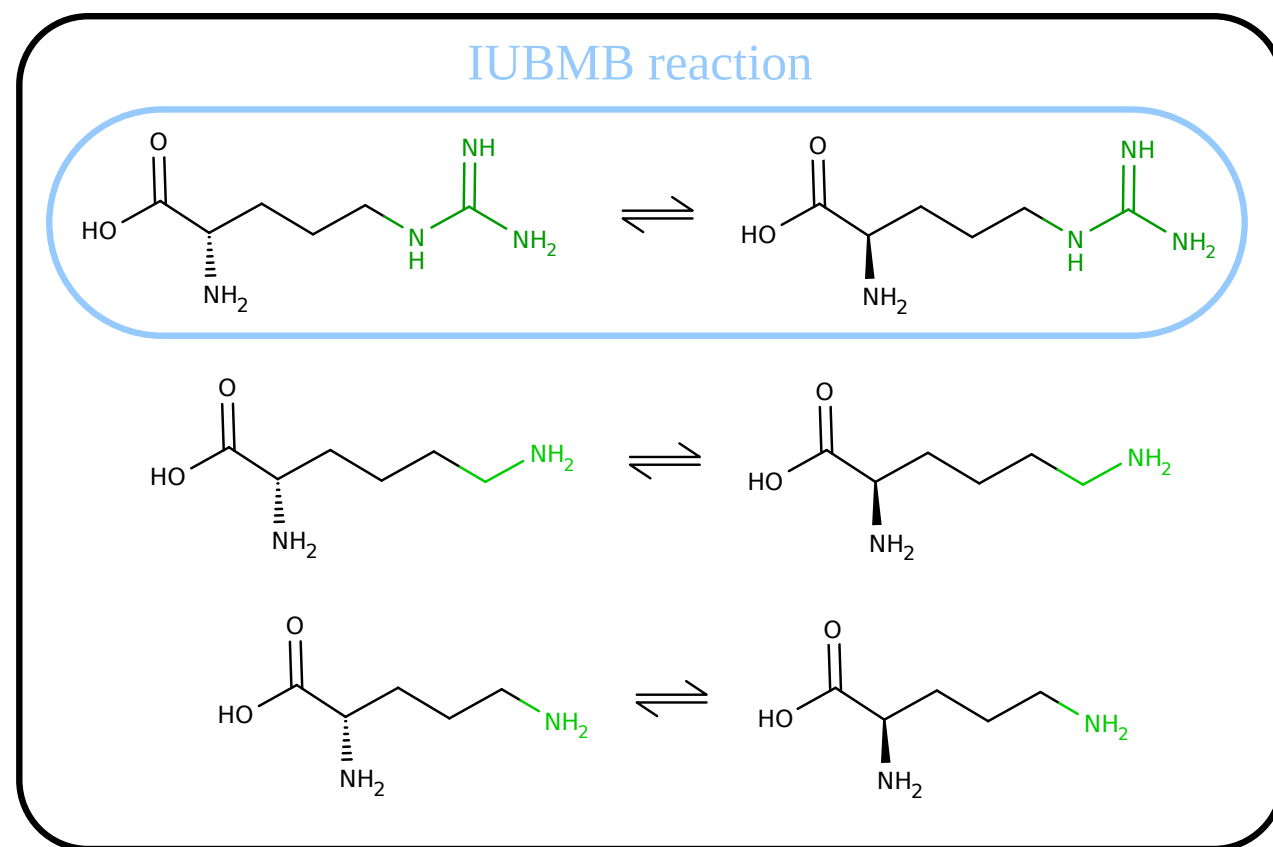


a

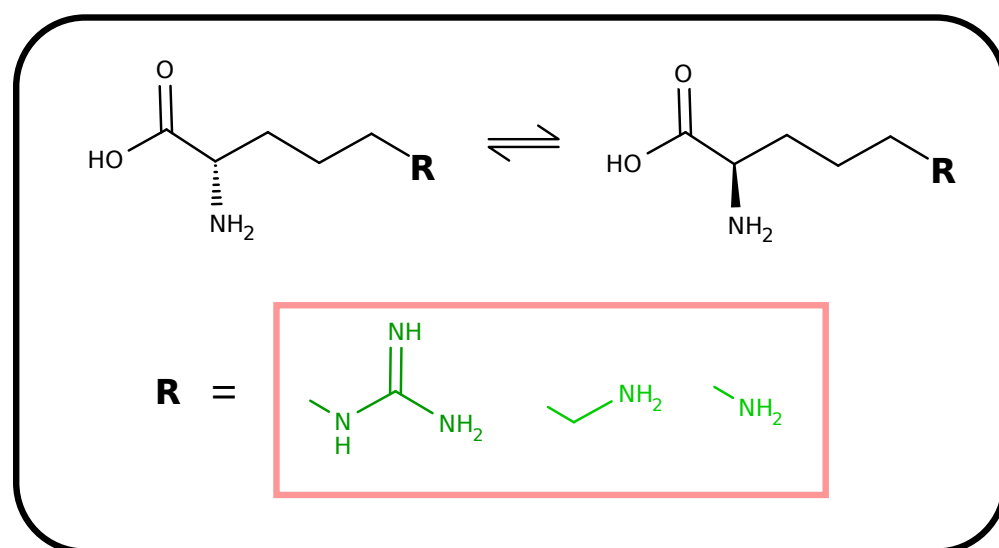
Collective

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Arginine racemase (EC 5.1.1.9)



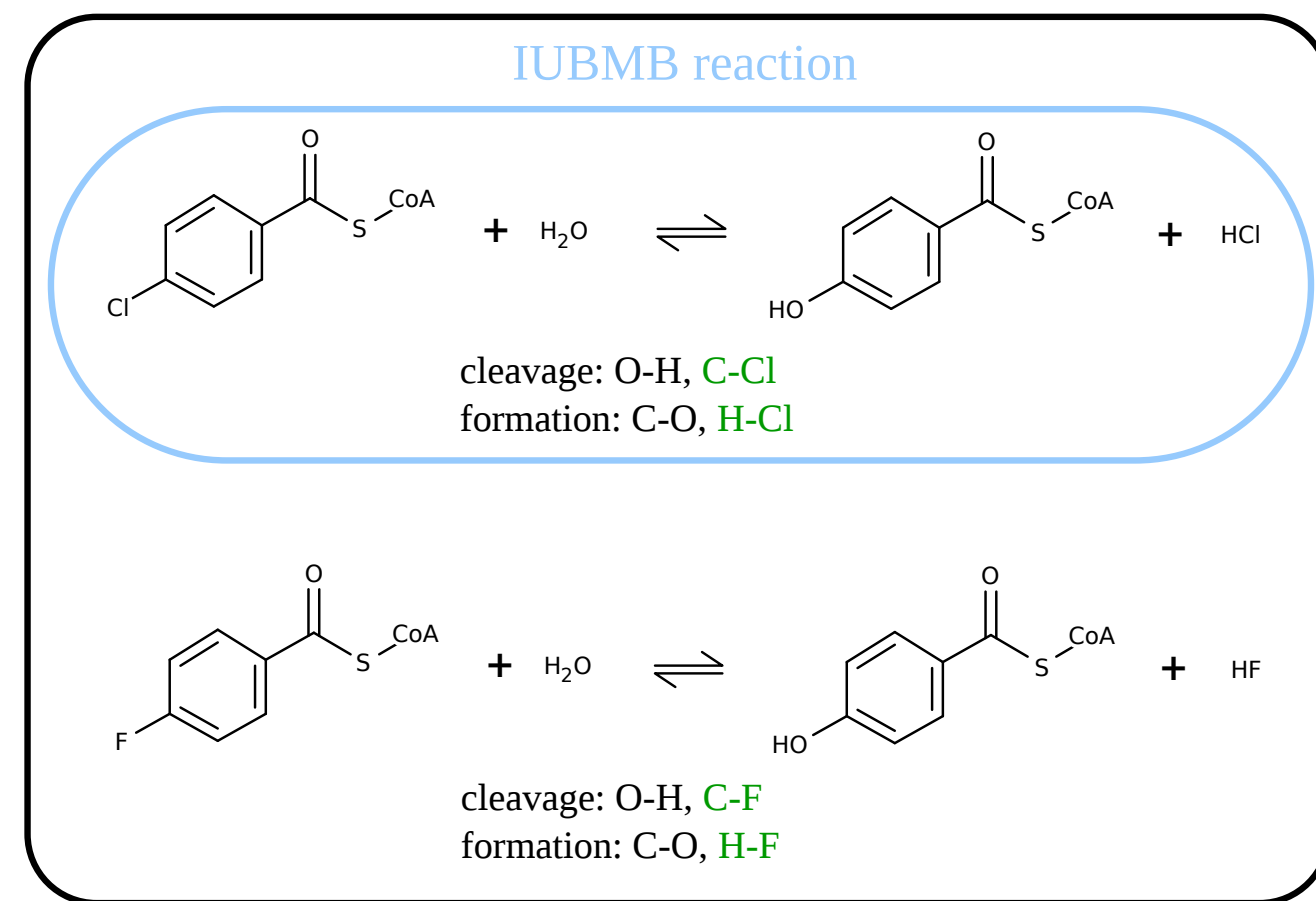
Arginine racemase (EC 5.1.1.9)



b

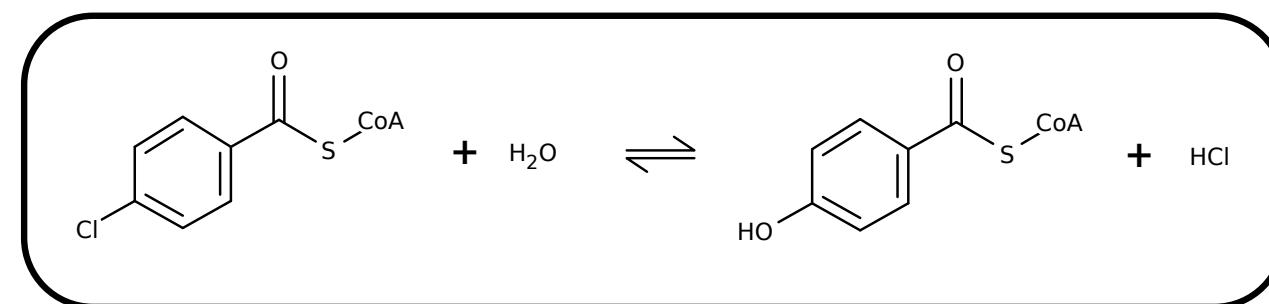
Specific

4-chlorobenzoyl-CoA dehalogenase (EC 3.8.1.7)



Split

4-chlorobenzoyl-CoA dechlorinase (EC 3.8.1.X)



4-fluorobenzoyl-CoA defluorinase (EC 3.8.1.Y)

