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Review

Initial catabolic steps of isoleucine, the *R*-pathway and the origin of alloisoleucine

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Abstract

The initial catabolic steps of isoleucine by mammals has been misunderstood and misapprehended in the scientific literature for many years. The suggestion that the interconversion of isoleucine and alloisoleucine occurs through the keto–enol racemization of their respective transaminated α-keto acids was first tentatively advanced by Alton Meister in the early 1950s, and accepted without hard confirming evidence by many authors. It will be shown in this brief review that isoleucine is converted to alloisoleucine with conservation of a ^{15}N label denying the intermediacy of the α-keto acids, and that alloisoleucine arises as an unavoidable consequence of isoleucine transamination.

Keywords: Reviews; Isoleucine; Alloisoleucine

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1. Early history

The essential amino acid L-(+)-isoleucine (2*S*-amino-3*S*-methylpentanoic acid) is one of two common protein amino acids that have two chiral centers (the other is threonine), and may therefore exist in four enantiomers. The second most commonly encountered enantiomer is L-(+)-alloisoleucine (2*S*-amino-3*R*-methylpentanoic acid). It has long been known that these two are slowly interconvertable in vivo, and many attempts have been made to illuminate the mechanism responsible. This amino acid system has a very interesting chemistry and a very colourful history.

Alton Meister observed in 1951 that rats fed a diet deficient in isoleucine but supplemented with 2-keto-3*R*-methylvaleric acid (*R*-KMVA) grew at a slower

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rate than those supplemented with *S*-KMVA or isoleucine [1]. Even though KMVA racemization was shown earlier [2] not to occur at pH<8.4, the suggestion was made that *R*-KMVA was converted to *S*-KMVA for reamination to isoleucine at a rate to allow at least slow growth.

The mechanism for this conversion, however, was not obvious and remained to be investigated [1]. Subsequently, many authors have assumed that keto–enol tautomeric racemization of KMVA enantiomers at physiological pH is responsible for the slow interconversion of isoleucine and alloisoleucine in vivo (Fig. 1), and that the enolic intermediate is the point in common between them.

The matter of there being two KMVA enantiomers possible has led to errors of presumption in the published literature. KMVA along with 2-keto-4-methylvaleric and 2-keto-3-methylbutyric acids, the keto acids derived by transamination of leucine and valine, respectively, are detectable by GC–MS of the trimethylsilyl (TMS) derivatives of the ketoximes made with hydroxylamine [3] in normal human serum and urine and in large elevations in those fluids in the inherited disorder α-ketonuria known trivially as maple syrup urine disease. As the TMS-oximes, KMVA produces two gas chromatographic peaks on achiral dimethylsilicone liquid phases having similar mass spectra (Fig. 2). The spectra are clearly similar ($M^{+\cdot}$=289 Da), but differ significantly in the relative intensities of the $M^{+\cdot}-CH_3^{\cdot}$ fragment at *m*/*z* 274. These two peaks have been mistakenly attributed to the resolution of the KMVA enantiomers in an achiral chromatographic system, which is impossible, when in fact they are resolvable by virtue of the non-chiral *E* and *Z* configurations of the oxime moiety itself (Fig. 3). Separation of the *E* and *Z* oxime isomers on a preparative scale can be effected by chelation with Cu^{2+} in aqueous solution [4]. The precipitated isomer is the chelate with the *E* oxime (the isomer in which the oxime OH function is oriented away from the carboxyl group). This demonstrated that the *E* and *Z* oxime TMS derivatives elute on dimethylsilicone in that order.

Nonetheless, *S*- and *R*-KMVA are entry points for the *S*- and *R*-pathways of isoleucine catabolism, respectively.

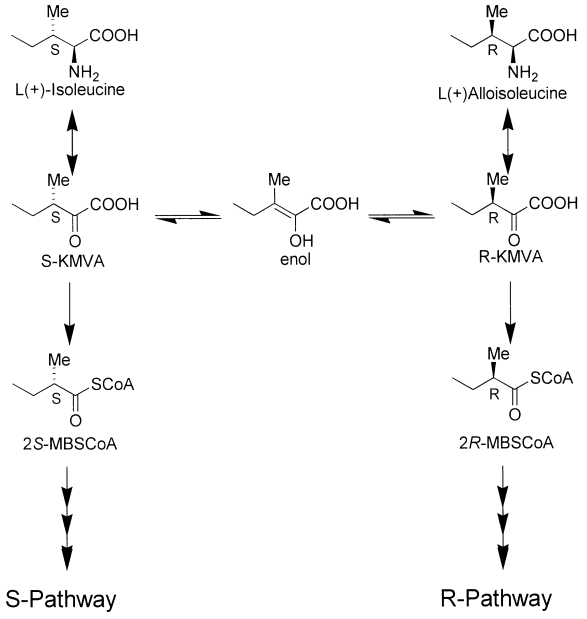


Fig. 1. The presumed and generally accepted mechanism for the interconversion of isoleucine and alloisoleucine. L-Isoleucine transaminase mediates the reversible conversion of isoleucine and alloisoleucine to *S*-KMVA and *R*-KMVA, respectively. Subsequently, branched-chain α-keto acid dehydrogenase irreversibly oxidatively decarboxylates the keto acids to produce 2*S*-methylbutyryl coenzyme-A (2*S*-MBSCoA) and 2*R*-methylbutyryl coenzyme-A (2*R*-MBSCoA) which are the first committed catabolites in the *S*- and *R*-pathways, respectively. In branched-chain α-ketonuria, this latter enzyme has reduced activity causing accumulations of the KMVA enantiomers.

2. The *R*-pathway

The *R*-pathway was discovered essentially by accident, the result of interpreting the mass spectrum of an unknown compound found elevated in the urine of the first patient described with inherited 2-methylacetoacetyl-CoA thiolase deficiency [5,6]. This unknown was shown to be the TMS derivative of 2-ethylhydracrylic acid (or 2-(hydroxymethyl)butyric acid, 2EHA) by comparison with a synthesized authentic sample, and was also found retrospectively in normal urine [7], as well as being elevated in the urine of a normal individual following an isoleucine load (Fig. 4). It has the carbon skeleton of many of the bacterial catabolites of isoleucine known at that time. Other metabolites found in that first urine were 2-methyl-3-hydroxy-

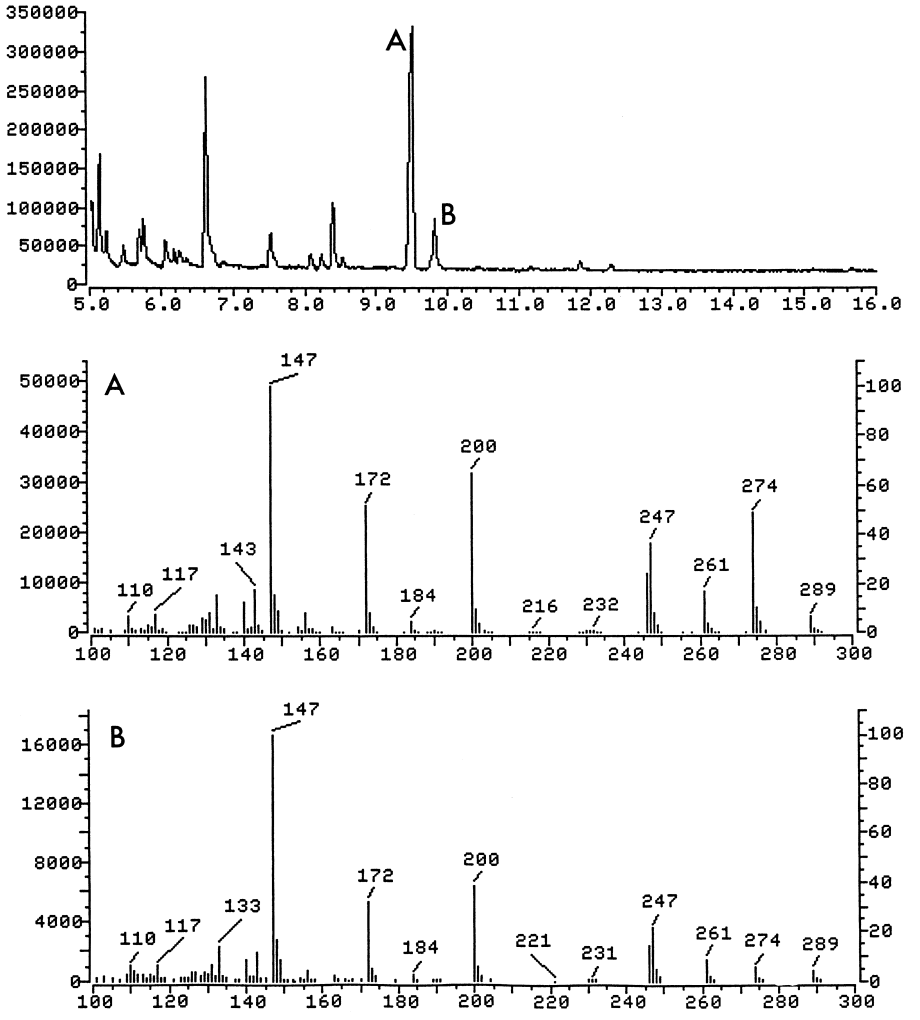
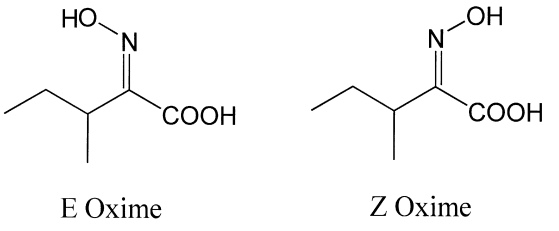


Fig. 2. GC–MS of the TMS derivatives of the oximes of authentic racemic KMVA. Two isomers are obtained, labeled A and B in the top panel, which have the spectra reproduced in panels 2 and 3, respectively. The analysis was done in electron impact on a HP-5988A GC–MS equipped with a 0.25-mm×30-m capillary column coated with a 0.25-μm DB-1 film and temperature programmed from 80° after a 1-min hold to 280° at 5°/min.



E Oxime Z Oxime

Fig. 3. Structures of the isomeric oximes produced by the reaction of hydroxylamine hydrochloride with racemic KMVA in alkaline aqueous solution.

butyric and 2-methylacetoacetic acids and *N*-tiglylglycine and were similarly identified by interpretation of the mass spectra of their TMS derivatives and synthesis of authentic samples for comparison. At that time these were known principally from bacterial metabolism studies.

Major steps in the *R*-pathway were delineated by the administration of several of the suspected intermediates to a variety of animals [8]. Gastric administration of 10 g of racemic 2-methylbutyric acid to a 17-kg dog, after at least partial esterification to

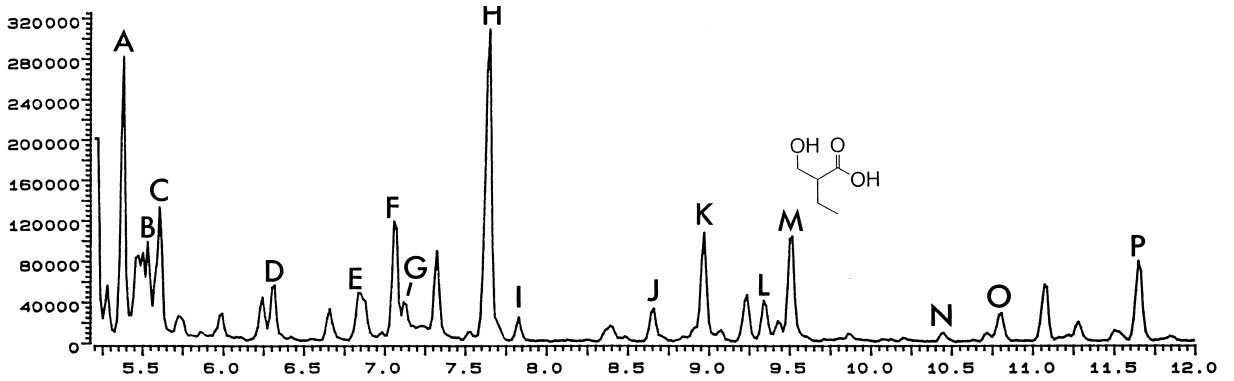


Fig. 4. Partial gas chromatogram of the TMS derivatives of the organic acids extracted from a normal human urine following an isoleucine loading. The eluting peaks are identified as the following acids: (A) lactic, (B) 2-hydroxyisobutyric, (C) glycolic, (D) oxalic, (E) 2-hydroxybutyric, (F) *p*-cresol, (G) hydracrylic, (H) co-eluting 3-hydroxyisobutyric and 3-hydroxybutyric, (I) 2-hydroxyisovaleric, (J) 2-methyl-3-hydroxybutyric, (K) 3-hydroxyisovaleric, (L) benzoic, (M) 2-ethylhydracrylic, (N) octanoic, (O) ethylmalonic, (P) succinic. Peaks not annotated are due to reagents and artifacts. The analysis was done under conditions similar to those described in Fig. 2.

coenzyme A in vivo, produced large increases in urinary 2EHA (up to 8000 mg/g creatinine, and still increasing after 2 h), and over 60 mg/l in serum. Gastric administration of racemic 2-[*Me*–$^{2}H_{3}$]methylbutyric acid to a rabbit produced very large increases in labeled urinary 2EHA and smaller increases in 2-[*Me*–$^{2}H_{3}$]methyl-3-hydroxybutyric acid. Intraperitoneal administration of resolved *S*-(+)-2-methylbutyric to rats produced only small increases in urinary 2-methyl-3-hydroxybutyric acid. On the other hand, very large increases in 2EHA were found following *R*-(−)-2-methylbutyric acid administration. Furthermore, 2-ethacrylic acid loading by i.p. injection to rats also produced major increases in the excretion of 2EHA. Examination of the isotopic content of 2EHA excreted by the rabbit following the load with racemic 2-[*Me*–$^{2}H_{3}$]methylbutyric acid showed it to be 3.8% unlabeled, 67.8% $^{2}H_{1}$ labeled and 28.4% $^{2}H_{2}$ labeled, demonstrating the reversibility of the oxidation of 2EHA to presumably 2-ethylmalonyl semialdehyde. Similar results were obtained in labeled experiments with rats [9]. The termination of the *S*-pathway is with the cleavage of 2-methylacetoacetyl-CoA by isoleucine ketothiolase to yield acetyl-CoA and propionyl-CoA, the step blocked in patients with 2-methylacetoacetyl-CoA thiolase deficiency. The termination of the *R*-pathway is unclear; intraperitoneal injection of racemic 2-methyl[3,3,4,4,4-$^{2}H_{5}$]butyric acid in rats yielded large concentrations of 2EHA that was 97% $^{2}H_{5}$-labeled in the ethyl group and small concentrations of [*Et*-$^{2}H_{5}$]ethylmalonic acid [8]. The latter could have been produced by direct oxidation of the labeled 2-EHA or by carboxylation of [3,3,4,4,4-$^{2}H_{5}$]butyryl-CoA produced by the action of 2-methylacetoacetyl-CoA thiolase on the semialdehyde.

Thus, 2*S*- and 2*R*-methylbutryic acids are the committed entry points to what has become known as the *S*- and *R*-pathways of isoleucine catabolism. The *R*-pathway may perhaps be more appropriately considered as the catabolic pathway for alloisoleucine.

It should be noted that the two pathways are characterized by catabolic steps that are analogous in terms of chemistry, but identical in terms of orientation in chiral space. For example, acyl-CoA dehydrogenase activity with 2*S*-methylbutyryl-CoA to produce tiglyl-CoA unsaturates the single bond that has the same orientation in space as the one in 2*R*-methylbutyryl-CoA which results in the appearance of 2-ethacrylic acid (Fig. 5). The same enzymes may be responsible for catabolizing both pathways.

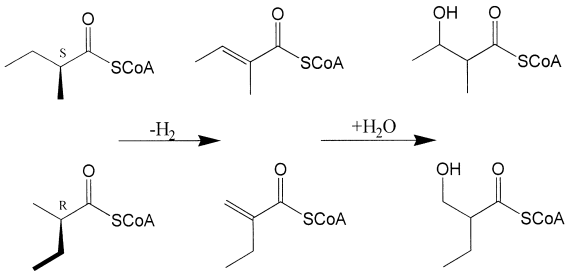


Fig. 5. Steps in the *S*- and *R*-pathways subsequent to those illustrated in Fig. 1. Unsaturation of the 2-methylbutyrate esters occurs in the same directed orientation in both enantiomers; in one case tiglyl-CoA (*S*-pathway) is produced, and ethacrylyl-CoA is the result of a similar process acting on 2*R*-methylbutyryl-CoA.

3. Keto–enol racemization

These two pathways have been thought for several decades to be related through an achiral enol intermediate produced from *R*- or *S*-KMVA. 2-Ketoacids are well known to be enolizable at high and low pH, and at that time it seemed reasonable to many authors [4,7–17] (this one included) that isoleucine and alloisoleucine were interconvertable through this means (Fig. 1).

The proposal of the enolic intermediate required KMVA to be spontaneously racemizable at physiological pH and temperatures. An attempt was made to estimate the rate of spontaneous racemization under these conditions by periodically measuring the intensity of the NMR signal developed by the H-3 in KMVA dissolved in $^{2}H_{2}O$ at neutral pD [18]. After 48 h at 37°C, the intensity of this proton resonance remained completely unchanged. Spontaneous enolization followed by re-ketonization would have replaced a significant fraction of the protium isotope in the 3-position with a deuterium from the solvent

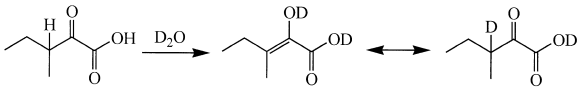


Fig. 6. Possible exchange of protium for deuterium labeling on carbon 3 of racemic KMVA in deuterium oxide through a putative enol intermediate at neutral pD.

(Fig. 6). This seemed to discount spontaneous KMVA enolization as a mechanism for the interconversion of isoleucine and alloisoleucine.

4. ^{15}N conservation

The *tert*.-butyldimethylsilyl (TBDMS) derivatives of the branched chain amino acids were analyzed by GC–MS in selected ion mode in urines collected from two rats loaded with small amounts of ^{15}N-labeled isoleucine which was also fully labeled in all carbon atoms with ^{13}C [19]. The ions monitored correspond to the loss of COO-TBDMS (*m*/*z* 200, 205 and 206) from alloisoleucine and isoleucine that are unlabeled (i.e., endogenous), derived from the load but ^{14}N-labeled, and derived from the load bearing the ^{15}N label, respectively. A second set of ions was monitored which correspond to the loss of C_4H_9 (*m*/*z* 302, 308 and 309) from the same amino acids, respectively (Fig. 7). Leucine, alloisoleucine and isoleucine elute in that order (Fig. 8). Leucine (retention time 7.07 min) produces a response in Fig. 8 only in the *m*/*z* 200 and 302 chromatograms as it does not have labeling above natural abundance. Alloisoleucine (retention time 7.33 min) elutes as a shoulder on the larger isoleucine peak (retention time

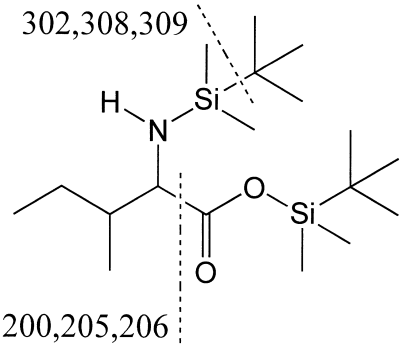


Fig. 7. Structures of the fragment ions of the TBDMS derivatives of urinary isoleucine and alloisoleucine selected for GC–MS monitoring following a loading of labeled isoleucine to rats. Fragments with masses 200 and 302 are unlabeled, fragments 205 and 308 are fully ^{13}C labeled, while fragments 206 and 309 additionally bear ^{15}N labeling.

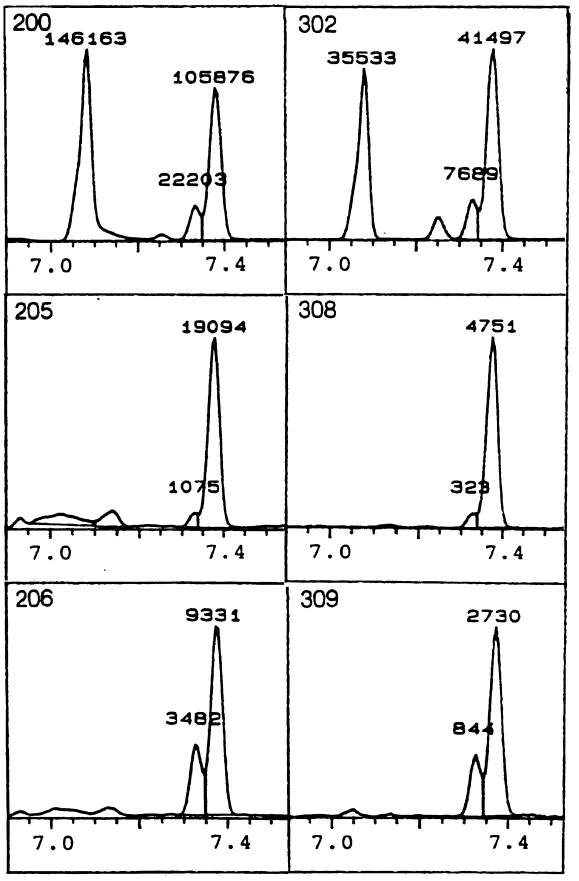


Fig. 8. Selected ion monitoring of the elution of leucine, alloisoleucine and isoleucine (eluting in that order) excreted by rats following the oral administration of [^{15}N, $^{13}C_6$]isoleucine. Peak areas were integrated by computer and are in arbitrary units. Analytical conditions were similar to those described in Fig. 2 with the exception that the temperature program was begun at 100° instead of 80°.

7.37). A relatively small response is produced by alloisoleucine derived from the load having exchanged the ^{15}N label for ^{14}N (m/z 205 and 308), while a considerably larger response is obtained for alloisoleucine having the ^{15}N intact (m/z 206 and 309). This demonstrates that the alloisoleucine derived from the load (fully ^{13}C labeled, to distinguish it in the presence of much larger concentrations of endogenous alloisoleucine) conserved most of the ^{15}N label (82 and 89% for the two rats) [19]. The remainder of the ^{13}C-labeled alloisoleucine which was ^{14}N labeled could have been made by reamination of [$^{13}C_6$]*R*-KMVA produced by transamination of nascent [^{15}N-$^{13}C_6$]alloisoleucine.

Conservation of ^{15}N label in this manner clearly denies any mechanism accounting for the interconversion of isoleucine and alloisoleucine in which the amino nitrogen is lost, such as KMVA keto–enol racemization. A proposal that meets this requirement is an adaptation of the mechanism accounting for alanine transamination [20,21].

This proposed mechanism (Fig. 9) provides for the eventual racemization of carbon 3 in isoleucine by having isoleucine (A1) condense with the aldehyde moiety of the pyridoxyl phosphate–aminotransferase enzyme complex (Py-Enz) to form an aldimine Schiff base (B1). Ketamine C1 is then formed by enzyme-mediated isomerization, in which the basicity of the nitrogen atom is much greater than that of the KMVA keto oxygen, and therefore able to easily

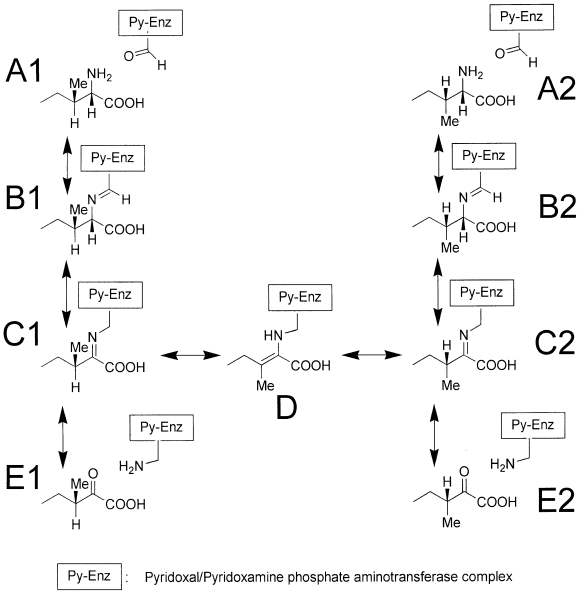


Fig. 9. A proposed mechanism consistent with and accounting for the conservation of labeled amino nitrogen in the conversion of isoleucine to alloisoleucine. Aldimine Schiff bases B1 and B2 are produced by the condensation of the amino acid amine groups with the aldehyde moieties of the pyridoxal phosphate–aminotransferase enzyme complex. These are enzymatically isomerized to the corresponding ketimines C1 and C2 in which the imino nitrogen is much more basic than the keto oxygen atom in KMVA, enabling the formation of the enolamine D, the intermediate in which the chirality of carbon 3 of the amino acids is lost. All the steps appear to be reversible.

form D, a ketimine (an enol analogue) in which the chirality of carbon 3 is lost. Reversion to ketamine C2 then enables release of either alloisoleucine (A2) by reversing the process through B2 or the release of fully deaminated *R*-KMVA (E2). Thus, alloisoleucine is formed from isoleucine without the obligate intermediacy of KMVA enantiomers, and is an unavoidable consequence of isoleucine transamination. In branched chain amino acid α-ketonuria [22], the oxidative decarboxylation of the α-keto acids is blocked causing, among other findings, large increases in 2*S*-hydroxy-3*R*-methylvaleric and 2*S*-hydroxy-3*S*-methylvaleric acids [18], isoleucine, *S*-KMVA and smaller but significant increases in *R*-KMVA and alloisoleucine [23,24]. Allowing for transaminative cycling between amino and keto acids, the opportunity for increased synthesis of alloisoleucine is clearly enhanced and accounts for not only the observed increase in alloisoleucine in this disorder, but also for the increase in *R*-KMVA [25]

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