Properties, biosynthesis, and catalytic mechanisms of hydroxy-amino-acids

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Abstract. The hydroxy amino acids have its unique effects in the biotechnology and molecular biology, and all sorts of synthetic hydroxy amino acids have also been developed. The hydroxy amino acids have been proved to be valuable for antifungal, antibacterial, antiviral and anticancer properties and known as a constituent of pharmaceutical intermediates. In addition to these fundamental researches, the hydroxy amino acids are applied widely to the synthesis of chiral drugs, such as (2S, 3R, 4S)-4-hydroxyisoleucine (4-HIL) is proved to be worthy in medical treatment, cis-4-hydroxy-L-proline (C4LHyp) and trans-4-hydroxy-L-proline (T4LHyp) are applied to build the chiral intermediates in pharmaceutical synthesis. Through comparisons chemical synthesis with biological catalysis to form hydroxy amino acids, enantioselective biocatalysts will undoubtedly be used in the synthesis of chiral pharmaceuticals. Mononuclear non-heme Fe(II)/α-ketoglutarate-dependent dioxygenases (Fe/αKGDs) that use an Fe(IV) complex intermediate to active diverse oxidative transformations with key biological roles. Studies identifying the important intermediates in catalysis and the proposed mechanisms are explained. In summary, we describe the physiological properties and synthesis regarding hydroxy amino acids, particularly 4-HIL and hydroxyproline. And the proposed catalysis mechanisms of Fe/αKGDs are also explained, while we also discussed the applications of hydroxy amino acids in fundamental research and industrial.

1. Introduction
Amino acids are pervasive chemical compounds of significant importance in the origin of life stem from their multiple critical functions in organisms. The hydroxylation of L-amino acids are an interesting and growing field of biotechnology and molecular biology research which have many physiological activities, for instance, that are the components of glycopeptide antibiotics, cyclodepsipeptides and collagen. These peptides contain hydroxy amino acid residues and are proved have properties of antifungal, antibacterial, antiviral and anticancer. Besides, some hydroxy amino...
acids could be utilized as blocks in the asymmetric synthesis of chiral drug [1].

There has been an increasing focus on the great potential of microorganisms and enzymes leading to the transformation of synthetic chemicals with high enantio-selectivities [2]. The enzyme-catalyzed has advantages over chemical synthesis with highly enantioselective and regioselective. Biocatalysis is a new developing environmentally-friendly technologies to obtain specific compounds, which could be carried out at room temperature and atmospheric pressure avoiding the utilize of more extremes. Microbial cells and enzymes catalysis can be immobilized and reused. Meanwhile, the enzymes can be obtained from inexhaustible biological resources, thus making biocatalytic processes economically efficient [3]. For example, sitagliptin, an antidiabetic drug is synthesized by the biocatalytic approach [4]. Compared with the chemical synthesis requiring heavy atoms (rhodium), organic solvent under high pressure and resulting in a yield of 97% of stereoselective compounds, biocatalytic approach only requires pyridoxal phosphate (PLP), transaminases under normal pressure and results in a yield of 99.95% of enantioselective compounds. In future, more enantioselective biocatalysts will undoubtedly be used in the synthesis of chiral pharmaceuticals. Moreover, enzymatic catalysis will greatly benefit in pharmaceutical manufacture.

Ferro iron and α-ketoglutarate dependent dioxygenases (Fe/αKGDs) were first identified in studies on collagen biosynthesis, where the Fe/αKGDs were found could catalyze the hydroxylation of prolyl and lysyl residues [5]. Following the pioneering work, dioxygenases are a very widely distributed family when it emerged. The dioxygenases catalyze a number of oxidation reactions, possibly the widest of any identified enzyme family. The dioxygenases were applied in business started with plant growth retardants and now extend to a clinical drug compound used for heart protection. Several dioxygenases are now explored to finish the target for therapeutic intervention for diseases including anemia, inflammation and cancer [6].

In this review, we describe the recent discoveries regarding the synthesis of physiological characteristics and microbial production of hydroxy-L-amino acids, particularly (2S, 3R, 4S)-4-hydroxyisoleucine and hydroxyproline. In addition to its properties the catalytic mechanisms of hydroxylase and applications in fundamental research and business are also discussed.

2. Synthesis and properties of hydroxy amino acids

2.1. The synthesis and properties of the 4-Hydroxyisoleucine (4-HIL)

2.1.1. The properties of 4-HIL. The 4-HIL, which was detected in the seeds of fenugreek firstly and that own 80 percent of total content free amino acid, the annual herb Trigonella foenum-graceum, has insulinotropic and anti-obesity effects and knowned as a promising drug for diabetes [7,8]. The 4-HIL has been used for the treatment of type II diabetes (e.g. sulfonylureas) as the insulin response mediated by 4-HIL that is directly based on the glucose concentration when compared with several types of pharmacological drugs. It is this unique property of 4-HIL that prevents adverse side effects such as hypoglycemia in the treatment of type II diabetes [9,10].

Besides, 4-HIL has more effect in regulating blood lipid and lowering cholesterol levels, on the one hand, the 4-HIL had efficacy on anti-hyperglycaemic and anti-dyslipidemic for the first time in a well-characterised model of type II diabetes in mice [11,12], on the other hand, it also could significantly decrease the levels of plasma triglyceride, total cholesterol, and free fatty acids in the dyslipidemic hamster model [13]. In summary, the 4-HIL should be a very promising treatment for prevention of chronic disease and its therapeutic benefits includes anti-infective, antioxidant, anti-inflammatory, cardioprotective and digestive stimulant activities [14-22]. Thus, the research of 4-HIL synthesis of receives more and more attention, urgent requirements for 4-HIL were also put forward by more and more people in modern times. The paragraph text follows on from the subsubsection heading but should not be in italic.

2.1.2. The synthesis of 4-HIL. Although several kinds of chemical and biological synthesis methods for
4-HIL have been reported, all in all, there are mainly three synthesis methods in industries including extraction and separation processes, chemical synthesis and biological enzyme synthesis (figure 1(a)) for the moment. Fenugreek (Trigonella foenum-graecum) is a promising medicinal herb. It has been used for many years and also applied to raw material for extraction of 4-HIL. Briefly, the amino acids were extracted by absolute ethanol, then the washed fractions were further purified by ion exchange resin, and the eluted fractions contain amount of the right 4-HIL, pure 4-HIL was obtain after further elution at last [23,24].

Figure 1. Typical biocatalytic synthesis of hydroxylated L-amino acids by Fe/aKG-DOs. (a) The C-4 hydroxylation of L-isoleucine forms L-4-hydroxyisoleucine (4-HIL). (b) The C-5 hydroxylation of L-leucine forms L-5-hydroxyisoleucine. (c) The C-4 hydroxylation of L-leucine generates L-4-hydroxyxyleucine. (d) The C-3 hydroxylation of L-arginine. (e) The C-4 hydroxylation of L-peoline forms 4S and 4R-hydroxyproline, respectively. (f) The C-3 hydroxylation of L-peoline forms 3S and 3R-hydroxyproline, respectively. (g) The C-3 and c-4 hydroxylation of L-lysine form L-3-hydroxylsine and L-4-hydroxylysine, respectively.
The chemical synthesis processes are often employed to chemical products and drug synthesis including ethyl-2-methyl acetoacetate, tert-Butyl bromoacetate, Ethyl 3-piperidinecarboxylate and so on. Wang et al provided an eight-step synthesis method as described for 4-HIL which has been achieved a 39% overall yield, one crucial step is the biotransformation of ethyl-2-methylacetoacetate to ethyl (2S,3S)-2-methyl-3-hydroxybutanoate by Geotrichum candidum [25]. There are another six-step chemical synthesis of 4-HIL with total control of stereochemistry, the final step being the biocatalytical preparation by hydrolysis of a N-phenylacetyl lactone derivative using the commercial penicillin acylase G, and the yield was up to 40% [26]. Arthrobacter simplex AKU 626 was found that could produce 4-HIL from acetaldehyde, α-ketobutyric acid, and L-glutamate by the function of the branched chain amino acid transaminase gene with yield of 3.2 mM 4-HIL [27].

The fully enzymatic synthesis of 4-HIL is to clone and highly express the bifunctional L-isoleucine-4-dioxygenase to provide active protein as a target for new drugs which seems to be getting faster, more efficient, therefore, this enzymatic synthesis strategy has been researched further and applied widely abroad as well as popular at home. Haefelé et al research results showed that isoleucine could be catalyzed forming 4-HIL by the dioxygenase on the presence of ferrous iron, 2-oxoglutarate (α-KG), ascorbate and oxygen, and it also indicated 2-oxoacid dependent dioxygenase has important roles in this biosynthetic pathway [28]. One bacillus thuringiensis strain 2e2 AKU 0251 that could hydroxylate the L-isoleucine to produce 4-HIL was screened, then the purified hydroxylase was measured, the results indicated it was a α-KG dependent L-isoleucine dioxygenase (IDO) which had high stereoselectivity to produce only (2S,3R,4S)-4-HIL [7]. This new study also provides a direction for developing microbial fermentations and biological catalysis to produce 4-HIL.

Based on the mechanism of the IDO hydroxylation process could couple the 2Δ strain destroyed TCA (figure 2), Smirnov et al cloned the IDO gene into Escherichia coli 2Δ strain which the α-KG dehydrogenase, isocitrate liase, and isocitrate dehydrogenase kinase / phosphatase were knocked out leading to a highly biotransformation of L-isoleucine into 4-HIL [24]. Shi et al modified the key enzyme 4-HIL dehydrogenase (HILDH) using protein modification strategies to enhance one-step carbonyl reduction to only produce (2S, 3R, 4S)-4-HIL, and this strategy achieved commercial-scale production of 4-HIL [29].

According to a comparison with three methods for 4-HIL (table 1), we could find that the extraction and chemical synthesis required more complex multilevel reactions, due to the complexity
of the products obtained by the two methods mentioned above, it was very difficult to isolate and purify the single 4-HIL, moreover, there is a struggle for control to stereoselectivity for 4-HIL. Thus, developing microbial fermentations and biological catalysis would become new direction to produce 4-HIL for industrialized application.

2.2. The synthesis and properties of hydroxyproline

Hydroxyproline has many isomers, which could produce in cis- or trans-forms, such as cis-3-hydroxy-L-proline (C3LHyp) and trans-3-hydroxy-L-proline (T3LHyp) (figure 1(e)), cis-4-hydroxy-L-proline (C4LHyp) and trans-4-hydroxy-L-proline (T4LHyp) (figure 1(f)). These hydroxyprolines exist in some proteins such as collagen, peptide antibiotics and plant cell wall, moreover, some isomers are used for the synthesis of pharmaceuticals as chiral building blocks.

2.2.1. The properties of T3LHyp and C3LHyp. T3LHyp is generated by prolyl 3-hydroxylase hydroxylation, which is the most plentiful in collagen. And another stereoisomer, C3LHyp has been realized the value as a drug to treat some diseases, such as cancer and collagen disorders, becoming the important biologically active components for chiral drug synthesis [30].

2.2.2. The synthesis of T3LHyp and C3LHyp. T3LHyp come mainly from the biosynthetic pathway, nevertheless, its biosynthetic pathway is still not clarified up to now as there are few researches to the proline trans-3-hydroxylase at present. However, C3LHyp has more understand as microbial production for pharmaceutical drugs [31]. C3LHyp is produced by a whole-cell biocatalyst, Johnston et al cloned the L-proline cis-3-hydroxylase gene from Streptomyces sp. TH1 into E. coli cells and was overexpress, then L-proline was put into the fermentation system as substrate, after cultivation 60 hours, the yield of C3LHyp could be up to 99% [32].

2.2.3. The properties of T4LHyp and C4LHyp. T4LHyp is formed by posttranslational modification, which is the most common in nature and key for the synthesis of procollagen, and it was provided to the cosmetics industry. C4LHyp is one ingredient of phallotoxin generated by the poisonous mushroom Amanita phalloides [33], which is effect on inhibiting the growth of new cells, meanwhile have also been applied to detect cells antitumor activity in tissue culture and in vivo [34]. In addition, C4LHyp has been used as an anticancer drug. [35,36].

2.2.4. The synthesis of T4LHyp and C4LHyp. The hydroxyproline could be synthesized by some chemical synthesis from D-glucose, D-mannitol, the synthesis method including conversion of D-glucose into N-benzyloxy carbonyl-c-alkenyl amine [37]. However, the hydroxyproline isomers, such as T4LHyp and C4LHyp, were not studied enough until enantioselective L-proline hydroxylases were

| Methods                               | Raw material                                 | Procedure feature                           | Product feature        |
|---------------------------------------|-----------------------------------------------|---------------------------------------------|------------------------|
| Extraction and separation processes   | Fenugreek                                     | Restrictions of local materials, complex    | Low-purity, difficult  |
| Chemical synthesis                    | Methyl 2-Ethylacetoacetate, acetaldehyde, α-KG and L-glutamate et al | Complex process, low yield, low efficiency for stereoselectivity of product | Low-safety, low-purity |
| Biological enzyme                     | L-isoleucine, L-isoleucine dioxygenase, α-KG et al | Efficient production, simple technique, Environment protecting | High-pure, high security |

Table 1. Comparison of 4-HIL industrial production methods.
The synthesis of T4LHyp had only been reported using method of biological catalysis [38]. Shibasaki et al used special strains to realize overexpression of L-proline-4-hydroxylase (L4HL) from Dactylosporangium sp. RH1 to obtain T4LHyp in proline oxidase-deficient cells [39]. Based on this method, many special strains such as Clonostachys cylindrospora, Gliocladium sp., and Nectria gliocladioides were chosen for producing T4LHyp in in an optimized medium [40,41]. Thus, to obtain more characteristic properties on the enzyme for catalysis T4LHyp, the L-proline-trans-4-hydroxylase (LT4HL) from S. griseoviridus P8648 has been studied for purifying and identifying in vitro evaluation. To improve the yield of T4LHyp, Shibasaki et al have explored to build commercial scale microbial production for T4LHyp [38].

The fully enzymatic synthesis of C4LHyp was obtained by using L-proline-cis-4-hydroxylase (LC4HL) to hydroxylates L-proline, and the LC4HL was discovered from rhizobia Sinorhizobium meliloti and Mesorhizobium loti [42]. In addition, the N-acetyl C4LHyp had also been produced from L-proline by LC4HL and N-acetyltransferase Mprl [43,44]. Bach et al designed that the conversion of LC4HL that came from rhizobia Sinorhizobium meliloti into C4LHyp from L-proline in vitro, which was evaluated to be approximately 10 % [43]. According to these reports we could find that the biosynthesis of C4LHyp utilized L-proline as initial material. However, in these cases, it was revealed that whether T4LHyp or C4LHyp synthesis were all relied on direct hydroxylation of L-proline by L4HL as the flexible conformation of hydroxyproline, and the biological catalysis method became worthy of study to produce hydroxyproline.

2.3. The synthesis and properties of L-5-Hydroxytryptophan
L-5-Hydroxytryptophan (5-HTP) is produced by L-tryptophan hydroxylase from L-tryptophan, which is an intermediate to synthesize serotonin. 5-HTP has been used for medical treatment as a precursor of the neurotransmitter serotonin for many years [45]. Thus, the 5-HTP has been used to treat some disease conditions, including in the therapy of depression, insomnia, fibromyalgia, obesity, antidepressant, chronic headaches [46-50].

At present, the main ways to obtain 5-HTP is extraction and separation processes from the seeds of the Griffonia simplicifolia [51], and which is commonly used in industry. The chemical and multienzymatic methods for synthesis of 5-HTP was reported by Winnicka et al, and the key intermediate was to obtain DL-alanine [52]. The multienzymatic synthesis of L-tryptophan and 5-HTP both in carboxylic group has been reported [53]. However, these methods of synthesis of 5-HTP have low yield, low-purity and difficulty in separation, therefore, the synthesis of 5-HTP need more research in the future.

2.4. The synthesis and properties of hydroxylysine
Hydroxylysine is naturally produced by non-proteinogenic β-hydroxy amino acids which are important component part of the peptides, protein scaffolds and antibiotics [54,55]. It is reported that hydroxylysine has two isomers, 3-hydroxylysine and 5-hydroxylysine (figure 1(g)), which were produced by enzymes catalysis and chemical synthesis, respectively [56,57]. Furthermore, 3-hydroxylysine and 5-hydroxylsine could be used for synthesis of 3-hydroxypipeolic acid and 5-hydroxypipeolic acid as an intermediary, respectively [58].

2.5. Other hydroxy-amino-acids
Although some other hydroxy-amino-acids were reported, there less researches work on it. 3-hydroxy-L-valine was found as synthesis intermediate of a HIV protease inhibitor, and that was important in the synthesis of HIV protease inhibitor [59]. (2S,3S)-3-hydroxyasparagine is a promising therapeutic for Gram-positive bacterial infections, and it also composes the calcium-dependent lipopeptide antibiotics [60]. (2S,3S)-3-hydroxyarginine (figure 1(d)) is used as biosynthesis of viomycin which is necessary for keeping the antimicrobial activity. In addition, this antibiotic is effective and valuable for the bacterial infections treat [61]. (2S,3R)-3-hydroxyleucine is broad development prospects
medical materials for the production of certain cyclic depsipeptides, and cyclic depsipeptides have function to be used as component of an antibiotic or platelet aggregation inhibitors [59], (2S,3R)-3-hydroxyphenylalanine is reported that found in the structure of lysobactin. (2S,4S)-5-hydroxyleucine (figure 1(b)) is refer to the biosynthetic pathway of (2S,4S)-4-methylproline, which is known to formation of nostopeptolides [62].

3. Catalytic mechanisms of ferrous iron [Fe(II)] and α-ketoglutarate (αKG)-dependent hydroxylase

It is the asymmetric hydroxylation that is still a significant reaction to synthetize valuable chiral compounds in the industry [63]. The Fe(II)/α-ketoglutarate-dependent dioxygenases (Fe/αKGDs) are applied urgently to design as amino acids hydroxylation biocatalysts for its highly regio- and stereoselectivity of the reactions [64]. And some hydroxylases belong to Fe/αKGDs which play an important role to specifically catalyze L-amino-acids producing hydroxylation of amino acids including 4-HIL, 5-Hydroxyleucine, (2S,3R)-3-hydroxyxyleucine, N-succinyl-(2S,3R)-3-hydroxyleucine, cis-4-hydroxy-L-proline, (2S)-4-hydroxyvaline [65], and so on. Fe/αKGDs are used to act on free amino acids with different types of regioselective hydroxylations. Thus, amino acids have been put to use as typical substrates of Fe/αKGDs to be converted into hydroxy amino acids.

In some cases, Fe/αKGDs are parts of multdomain proteins which is in accordance with catalysis mechanism. The substrates hydroxylation is typical reactivity of Fe/αKGDs and is also researched most frequently and deeply. The functions of Fe/αKGDs contain to repair of DNA/RNA alkylation damage, demethylation of 5-methylcytosine and degrade environmental substances [5,66-68]. The hydroxylation process needs Fe(II) and α-KG as cofactors. Oxygen is required in the hydroxylation to oxidatively break down α-KG into succinate and CO₂ while another oxygen atoms attacks the substrate to form hydroxy amino acid. The Fe/αKGDs have common structural features, including a typical protein fold known as double-stranded b-helix (DSBH) fold, an HXD/EXnH iron binding motif that is the core of the protein structure [68-70]. The α-KG binds to the iron in a bidentate manner via its 1-carboxylate and 2-oxo groups and its binding sites are relatively conserved. However, it is variation of the substrate-binding sites, leading to the hydroxylation has highly stereoselectivity and substrate specificity.

In all reported structures of the enzyme Fe/αKG-DOs complex, Hanuske-Abel et al [71] first put forward a mechanism involving the special relations and coordination of three metal-amino acid side chains in the active metallocentre and generating a valent iron: oxygen atom intermediate. After that, many other Fe/αKGDs structures were found which permitted redefinition of this structure model and producing a basic common mechanism as shown in figure 3. The catalysis starts with Fe(II) attached to the 2-His-1-carboxylate facial triad [72,73], and the other three coordination sites occupied by water molecules (figure 3(a)). Then the α-KG binds to the Fe(II) active metallocentre in a bidentate configuration, with its keto group and carboxylate opposite the structure of 2-His-1-carboxylate facial triad, which results in replacing two metal-bound water molecules (figure 3(b)). Following α-KG binding, it is the binding of the remaining water to the metal was weakened by substrate-triggered leading to substrate can be easy to bind the active coordination site displacing the third metal-bound water molecule (figure 3(c)). The substrate binding process triggered an oxygen molecule to bind Fe(II) active center and occur oxidation reactions with generating an Fe(III)-superoxo intermediate [74] (figure 3(d)). Then 2-ketogroup of α-KG are attacked by the distal oxygen atom of the Fe(III)-superoxo species leading to generating a Fe(IV)=O species (figure 3(e)). Soon to come is oxidative decarboxylation of α-KG triggered by Fe(IV)=O species, and then the CO₂ is released [75], the ferryl intermediate and succinate are generated (figure 3(f)). The last second step is that a substrate radical and Fe(III)-OH species are formed after Fe(IV)-oxo species acquired a hydrogen atom from the substrate (figure 3(g)). Lastly, the hydroxyl radical rebound (or more complex chemistry) completes substrate hydroxylation and recycles the enzyme to the initial Fe(II) state [76].
Figure 3. Proposed mechanism of reaction by Fe/αKG-DOs. Middle, Structure of a representative Fe/αKG-DOs (PDB access code 1GY9). TauD is constructed with red α helices, yellow β strands, green unstructured regions, α-KG (sticks with yellow carbons), Fe (orange sphere) and side chains are integrated in stick mode, furthermore the position of the metallocentre relative to the major group (double-stranded β helix core). The (a)-(g) is the simplified hydroxylation mechanism.

The above proposed hydroxylation mechanism was supported by extensive structural studies of Fe/αKGDs that provides experimental data [68,77-79]. And some crystal structures of Fe/αKGDs revealed the consensus facial triads and Fe(II) active center coordination environments as shown in the figure 3. The decarboxylation of α-KG almost is not accompanied with the substrate oxidation that has been studied in many Fe/αKGDs [79]. This substrate ‘uncoupled’ turnover with α-KG could be observed in many cases, such as AlkB and TauD, consequently leading to aromatic residues auto-oxidation in hydroxylation process which may also be mediated by the reactive Fe(IV)=O species [66]. Tuderman et al found [80] that the ascorbate has function of decreasing the oxidation of Fe(II) regenerating the catalytically active Fe(II) species in resting state.

4. Conclusions and perspectives
Hydroxy amino acids are essential and important kind amino acids in the case of its biological functions and biotechnological cosmetic applications. It has physiological activities and function of antifungal, antibacterial, antiviral and anticancer properties, but can be used as precursors belong to
important components of chiral compounds in the asymmetric synthesis of pharmaceuticals. A unique conformation of hydroxy amino acids contributes to the formation of chemical isomers, result in the generation of various hydroxy amino acid analogues. As described previously, 4-HIL has effect on for prevention of chronic disease; and hydroxy-L-proline are also used as medical intermediates for pharmaceutical. Due to the emergency hydroxyproline isomers, it has different effect on biological functions, such as C3LHyp, T3LHyp, C4LHyp and T4LHyp. Nowadays, chemical synthesis has been developed for many hydroxy amino acids synthesis including unnatural compounds, such as 4-HIL and some hydroxyproline. And some other hydroxy amino acids found in biological resources could be obtained by extracted process. However, biological catalysis with existence of related hydroxylase could produce 4-HIL, peptide-like compounds containing hydroxyproline and so on. The biocatalysis of some L-amino acids is poorly studied and needs more comprehensive exploration. The Fe/αKGDs has a prominent ability to trigger the oxygen molecular for realize the important oxidative transformations and the oxidative decarboxylation of the α-KG moiety of the substrate to generate the key ferryl intermediate [80]. Furthermore, new members of the Fe/αKG-DOs superfamily is certain to be discovered in the biosynthesis of hydroxy-L-amino acid, and the research and application of hydroxy-amino acid would be more and more extensive in the future.

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