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N-Alkyl- α -amino acids in Nature and their biocatalytic preparation

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#Equal contribution

Highlights

- The *N*-alkyl amino acid moiety is widespread in Nature.
- A number of commercial products related to health contain the *N*-alkylated amino acid moiety.
- Biocatalysis can provide more efficient and sustainable methods of accessing the *N*-alkyl amino acid moiety than traditional methods.
- Several new enzyme classes comprising *N*-methyl transferases and dehydrogenases can be used for the synthesis of *N*-alkyl amino acids.

Abstract

N-alkylated- α -amino acids are useful building blocks for the pharmaceutical and fine chemical industries. Enantioselective methods of *N*-alkylated- α -amino acid synthesis are therefore highly valuable and widely investigated. While there are a variety of chemical methods for their synthesis,

they often employ stoichiometric quantities of hazardous reagents such as pyrophoric metal hydrides or genotoxic alkylating agents, whereas biocatalytic routes can provide a greener and cleaner alternative to existing methods. This review highlights the occurrence of the *N*-alkyl- α -amino acid motif and its role in nature, important applications towards human health and biocatalytic methods of preparation. Several enzyme classes that can be used to access chiral *N*-alkylated- α -amino acids and their substrate selectivities are detailed.

Keywords

N-Alkyl- α -amino acids; Biocatalysis; *N*-Methyl transferases; Dehydrogenases

1. The occurrence of *N*-alkyl- α -amino acids

N- α -Alkylation represents one mechanism by which nature can modulate the activity of chiral α -amino acids. The most abundant are *N*-methyl- α -amino acids, encountered sometimes as the monomer, but more frequently, embedded within complex peptide or non-peptide natural products. Perhaps the most well studied natural *N*-methyl- α -amino acids are sarcosine (1) (*N*-methyl- α -L-glycine), *N,N*-dimethylglycine (2) and betaine (3) (also known as glycine betaine) (Fig. 1). All three metabolites are ubiquitous in nature, produced by plants, animals and microorganisms. Each of these metabolites has valuable biological properties that have been the subject of much interest for potential commercial exploitation. Sarcosine (1), currently used as a dietary supplement and as a non-specific glycine transport inhibitor, also holds promise as an adjuvant therapy with sodium benzoate for the treatment of schizophrenia (Lin et al., 2017) and depression, as recently discussed in a review (Mathew, 2013). *N,N*-Dimethylglycine (2), which is also used as a dietary supplement, reportedly improves immune responses, acts as an athletic performance enhancer, displays anticonvulsant activity and may have use as an antidepressant in patients with autism (Lee et al., 2017). According to a recent review by Wilcken, glycine betaine (3) is used pharmaceutically in anhydrous form as Cystadane®, an adjunctive treatment for homocystinuria and can offer numerous other benefits to human health such as attenuation of liver injury (Wilcken, 2017).

A broad range of betaines, of which glycine betaine (3) represents just one example, exist in nature and their main function is to act as osmolytes. They are produced in particularly high abundance (up to 4M concentrations) by organisms such as cyanobacteria, lichen, marine algae and salt tolerant plants (Chambers and Lever, 1996). Some examples of naturally occurring betaines include the marine indole alkaloids 5,6-dibromo-L-hypaphorine (4) and plakohypaphorine D (5), which also display antioxidant and antihistaminic activity respectively (Netz and Opatz, 2015).

Ribosomal peptides are of huge importance to the pharmaceutical industry, representing over sixty marketed drugs (Wegmuller and Schmid, 2014; Zompra et al., 2009). These range from short, chemically synthesised peptides of up to twenty residues, to peptides of up to 100 residues that are typically produced using recombinant DNA technology in mammalian, yeast or bacterial host systems. Ribosomal peptides and proteins frequently undergo post-translational modification via a broad variety of transformations (Nesterchuk et al., 2011) and the so-called ribosomally-synthesized and post-translationally-modified peptides (RiPPs) have recently been comprehensively reviewed (Arnison et al., 2013). Post-translational modification at the α -nitrogen atom of *N*-terminal amino acid residues of linear ribosomal peptides (*N*- α -modification) is a common example of post-translational modification, typically occurring through *N*-acylation and less commonly through *N*-methylation (Ann et al., 1987). The *N*-methylation of side-chain residues of histone proteins has been extensively studied over the past 20 years (Murn and Shi, 2017), whereas the role of methylation in other proteins and peptides, including *N*- α -methylation, is still largely unknown (Clarke, 2013; Erce et al., 2012). However, many of these peptides appear to function by interacting with proteins, often as part of extensive macromolecular complexes such as the epigenome and interactome.

N- α -Methylation can have a profound effect on the biological activity of peptides. For example, cypemycin is a naturally occurring bacterial peptide that contains post-translational modifications, including *N*- α -dimethylation and displays potent antimicrobial activity and *in vitro* activity against mouse leukemia cells. Unlike cypemycin, its demethylated counterpart, prepared biosynthetically by deletion of the gene responsible for methylation, was inactive when tested towards growth inhibition of the gram-positive bacterium *Micrococcus luteus* (Claesen and Bibb, 2010). In contrast, with the intention of improving the stability of the antimicrobial peptide Nisin, which is used widely in the food industry, tandem *N*- α -monomethylation and lysine side-chain dimethylation, using the *N*-methyltransferases (NMT) from the cypemycin biosynthetic pathway (CypM), resulted in a trimethylated derivative that displayed a 4 and 8-fold increase in minimum inhibitory concentration (MIC) towards *Lactococcus Lactis* HP (ATCC11602) and *Bacillus subtilis* 168 (ATCC6633) respectively (Zhang and van der Donk, 2012).

Cyclic peptides are important in medicinal chemistry because they offer a number of advantages over acyclic peptides such as improved stability towards proteases, better membrane penetration and reduced toxicity due to enhanced receptor selectivity resulting from reduced conformational flexibility (Ripka et al., 1998). Cyclosporin A (6), which contains seven backbone *N*-methyl groups, represents an excellent example of a conformationally constrained cyclic non-ribosomal peptide (NRP). Approved by the FDA in 1983, it was still one of the top 100 selling brand name drugs in 2016 (Smith et al., 2016), marketed by Allergan under the trade name Restasis.

N-Methyl- α -amino acids are also found embedded into small non-peptoidal secondary metabolites. For example, 4-dimethylallyl-L-abrine (7) is a key intermediate in the biosynthesis of ergot alkaloids (Fig. 1) (Rigbers and Li, 2008).

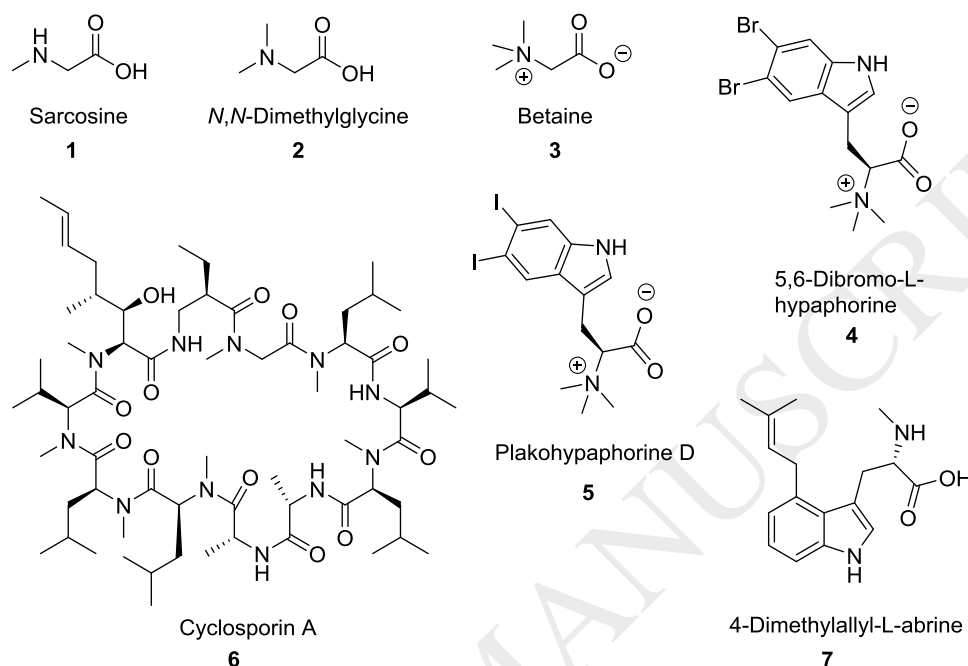


Fig. 1. *N*-Methylated glycine primary metabolites of the folate cycle.

Alkyl groups other than methyl can also be naturally incorporated into *N*-alkyl- α -amino acids. For example, opines are produced by a wide variety of organisms including bacteria, plants, and animals including humans (Kung and Wagner, 1970; Schrittwieser et al., 2015).

The primary role of opines in many organisms is in anaerobic respiration (Grieshaber et al., 1994). In fact, the opine pathway is thought to be the oldest of four anaerobic respiration pathways, resulting from the high amino acid abundance and low oxygen levels present in primordial times and is still of key importance to organisms, such as marine invertebrates, that inhabit hypoxic environments (Harcet et al., 2013; Livingstone et al., 1983). There has also been extensive investigation into the role of opines in plant cancers, such as crown gall and hairy root disease, where pathogenic bacteria genetically modify plant cells by transferring bacterial plasmids to the plant chromosome, thereby facilitating the production of opines for use as nutrients and reducing microbial competition (Dessaux et al., 1993). The ability of these bacteria to transfer DNA to other organisms has met with significant interest, whereas, to the best of our knowledge there is little information on the bioactivity of opines or opine derivatives themselves.

A number of different opine families exist, most having a structure resulting from the reductive amination of an α -amino acid with an α -ketoacid, as typified by octopine (8). However, there are many other classes, such as opines that utilise alternative keto acids, or that utilise sugars rather than keto acids (Fig. 2) (Dessaux et al., 1993). One particularly interesting aspect of opines is that the majority contain one amino acid of common L-(*S*)-stereochemistry and the other with D-(*R*)-stereochemistry, where the unusual D-(*R*)-stereocentre is formed during the enzyme catalysed condensation reaction. However, L,L-(*S,S*)-opines, such as L,L-(*S,S*)-succinamoprine, have also been identified (Chilton et al., 1985). A more complex example is that of a new class of bacterial opine metallophores, such as staphylopine (9) produced by *Staphylococcus aureus*, that play a key role in pathogenesis by sequestering metals from their host (Song et al., 2018). These are biosynthesised from three amino acids by the action of *R*-selective 3-amino-3-carboxypropyltransferase (nicotianamine synthase) catalysed *N*-alkylation followed by *S*-selective coupling of the resultant product to a further α -ketoacid by an opine dehydrogenase (McFarlane et al., 2018; McFarlane and Lamb, 2017).

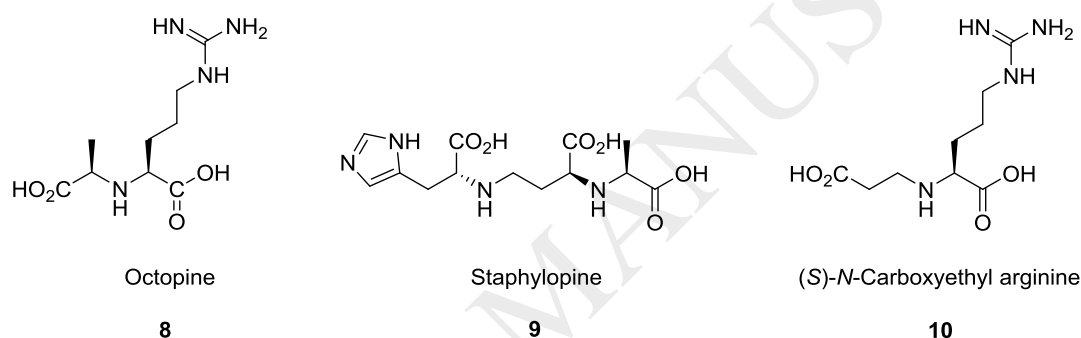


Fig. 2. Some examples of naturally occurring opines.

Other *N*- α -alkyl groups of amino acids seem to be quite rare or are perhaps embedded into biosynthetic pathways that have yet to be elucidated. One such example that has been identified is (*S*)-*N*-carboxyethyl arginine (10) which is an intermediate in the biosynthesis of clavulanic acid (Elson et al., 1993), a commercial β -lactamase inhibitor that is frequently administered with penicillin antibiotics to minimise drug resistance. This unusual functionality is introduced by the rare thiamine diphosphate (ThDP) dependent carboxyethylation of arginine by the N^2 -(2-carboxyethyl)arginine synthase (CEAS) enzyme (Caines et al., 2009).

2. Preparation of *N*-alkyl- α -amino acids

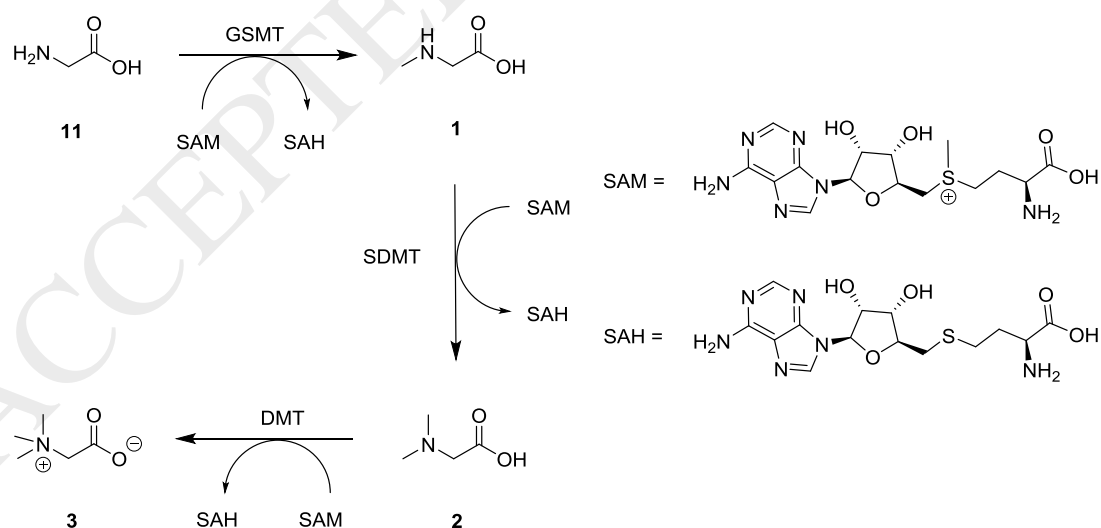
The chemical synthesis of *N*-alkyl- α -amino acids is usually performed by *N*-alkylation processes, which employ genotoxic alkylating agents, or reductive amination, utilizing stoichiometric quantities of hazardous hydrides which requires complex work-up procedures and the generation of significant waste (Aurelio et al., 2004; Aurelio and Hughes, 2009; Baxter, 2004; Chruma et al., 1997). Various asymmetric reductive amination methods have also been reported (Arceo and Melchiorre, 2012; Chen

et al., 2009a; Kuang and Distefano, 1998; So et al., 2012), but they each suffer from limitations such as narrow substrate range, use of heavy metals, or the requirement to pre-form the imine to avoid ketone reduction (Aurelio et al., 2004). Biocatalysis offers a less hazardous and wasteful alternative to traditional chemical synthetic methods. Therefore, given the wide variety of natural products that contain *N*-alkyl- α -amino acids, it is not surprising that many enzyme classes, such as *N*-methyltransferases and several dehydrogenases, have the potential to be used for *N*-alkyl- α -amino acid synthesis. The use of these enzymes for *N*-alkyl- α -amino acid synthesis is an emerging field and is the focus of this section.

2.1. *N*-methyltransferases

N-Methyltransferases (NMT, EC 2.1.1), are ubiquitous in nature and are responsible for the methylation of a broad range of natural products including the *N*- α -methylation of many of the amino acids, peptides and proteins mentioned above.

These enzymes are highly versatile and depending on their function, can be either highly substrate selective or promiscuous, producing mono-, di- or trimethylated products. For example, in mammalian systems, sarcosine (1) is produced by the selective mono-methylation of glycine (11) by *S*-adenosylmethionine (SAM) dependent glycine sarcosine methyltransferase (GSMT), whereas *N,N*-dimethyl glycine (2) and glycine betaine (3) are only accessible from choline (Ducker and Rabinowitz, 2017). A similar pathway is followed in most other organisms, but there are some rare examples of bacteria, that inhabit hypersaline environments, which can produce glycine betaine (3) via a sequential 3-step methylation of glycine using GSMT, sarcosine dimethylglycine methyltransferase (SDMT) and dimethylglycine methyltransferase (DMT) respectively (Scheme 1) (Zou et al., 2016).



Scheme 1. Biosynthesis of glycine betaine from glycine by sequential methylation that is known to occur in some halophilic bacteria.

A few examples of broad substrate spectrum NMTs, that are capable of direct conversion of amino acids to betaines, have also recently been reported. For example, histidine betaine, an intermediate in the biosynthesis of ergothioneine, is produced directly from histidine in *Mycobacterium smegmatis* solely by the histidine betaine synthase, EgtD (Vit et al., 2015). Unfortunately, EgtD only accepts histidine as substrate, but genome searching identified numerous homologues in bacterial and fungal genomes. Based on this search, the EgtD homologue from *Aspergillus nidulans*, recombinantly expressed in *E. coli*, showed a preference for tyrosine, although histidine, dihydroxyphenylalanine and phenylalanine were tolerated with lower efficiency. In contrast, the halophilic methanoarchaeon *Methanohalophilus portucalensis* strain FDF1^T contains NMTs capable of both stepwise and direct glycine betaine formation (Chen et al., 2009b). Examples of individual NMTs involved in the stepwise conversion and direct conversion of amino acids to betaines have also been heterologously expressed in *E. coli* in an active form (Lai and Lai, 2011).

NMTs involved in the terminal *N*- α -methylation of peptides and proteins are common and are usually promiscuous towards the peptide sequences they accept, often *N*- α -methylating a range of peptides to different extents and sometimes their sidechains as well. For example, CypM, which is the NMT responsible for the introduction of the *N*- α -dimethylation functionality of cypemycin has been demonstrated to methylate a range of short heptapeptides that resemble the *N*-terminus of the natural substrate, as well as the unrelated antimicrobial peptides, nisin and halodracin (Zhang and van der Donk, 2012). However, this is not always the case as demonstrated by the very selective PZN methyltransferase. This enzyme is highly selective towards the *N*- α -dimethylation of the highly post-translationally modified peptide desmethylplantazolicin to afford plantazolicin (PZN), an antibiogenic peptide with bacteriocidal activity toward strains of *Bacillus anthracis*, the causative agent of anthrax (Lee et al., 2013).

In contrast to ribosomal peptides, NRPs are backbone *N*-methylated by methylation domains at the terminal *N*- α -position of amino acids or peptides, attached to the same non-ribosomal peptide synthase (NRPS) at peptidyl carrier protein (PCP) regions through thioester linkages (Mori et al., 2018). However, with the aim of improving titres, a second mechanism was recently found when investigating the biosynthetic pathway to the fungal metabolite cycloaspeptide E5 (12) that displays interesting insecticidal activity (de Mattos-Shiple et al., 2018). Unexpectedly, the NRPS required directly incorporates *N*-methyl phenylalanine that is pre-formed by an NMT. This allowed the titre of (12) to be greatly increased by direct feeding of *N*- α -phenylalanine to the fermentation. Unfortunately, the NRPS accepted a very narrow range of alternative substrates, although *N*-methyl-para-fluorophenyl alanine was accepted, allowing access to the difluorinated analogue (13) (Fig. 3). Searching the NCBI database for homologues of the cycloaspeptide E NMT gene, *PscyA*, the same authors rapidly identified a homologue involved in the biosynthesis of the fungal metabolite ditryptophenaline (14). Taking the

same feeding approach, a difluorinated analogue of ditryptophenaline (15) was also prepared, implying that numerous fungal NMTs capable of *N*- α -methylating amino acids probably exist.

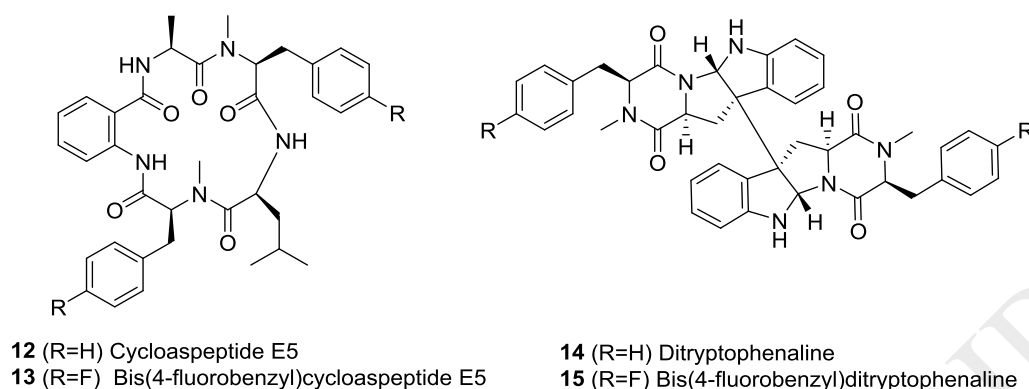


Fig. 3. Some NRPs and their fluorinated analogues that can be prepared through introduction of intact *N*- α -methylamino acids to the fermentation process.

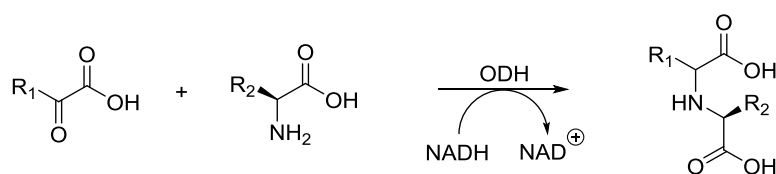
SAM and its analogues are expensive to use in stoichiometric quantities, which has hampered the use of NMTs in in-vitro preparative biotransformations. This has led to methods of in-situ generation from cheap materials, such as the tandem use of Sall, an enzyme which catalyses the alkylation of 5'-deoxy-5-chloroadenosine with methionine and its *S*-alkyl analogues (Sadler et al., 2017). Whereas the use of these approaches is still at an early stage, they hold much promise for the future as methods of green and sustainable *O*-, *N*- and *C*-alkylation.

2.2. Dehydrogenases

While there are many enzymes which can carry out reductive amination using ammonia, few examples are known to employ alkyl amines (Hummel and Groger, 2012; Schrittwieser et al., 2015; Sharma et al., 2017). Classes of enzymes that are now known to perform this transformation include opine dehydrogenases (ODHs), *N*-methyl amino acid dehydrogenases (NMAADHs), ketimine reductases (KIREDS), pyrroline-5-carboxylate reductases (P5CRs) and imine reductases (IREDS), each of which is discussed below.

2.2.1. Opine dehydrogenases

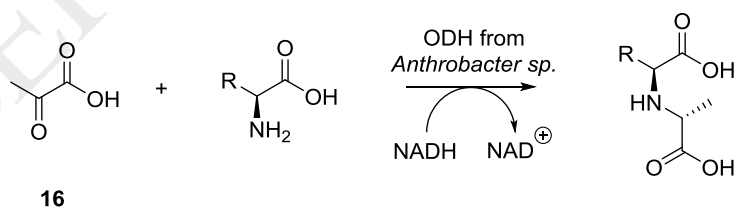
Opine dehydrogenases (ODHs) are oxidoreductases whose natural role is to catalyse the reductive amination of α -keto acids with amino acids to provide *N*-carboxylalkyl amino acids (Scheme 2), a class of compounds known informally as opines. Octopine dehydrogenase (OcdH, EC 1.5.1.11) was the first ODH to be identified in 1959, when Van Thoai et al. synthesized D-octopine (8) *in vitro* from arginine and pyruvic acid in the presence of various enzyme preparations from marine invertebrates (Van Thoai and Robin, 1959). A decade later the same group purified the NADH dependent OcdH from mussels of *Pecten maximus* (Van Thoai et al., 1969).

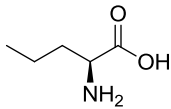
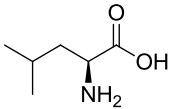
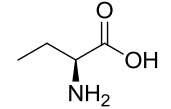
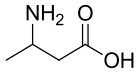
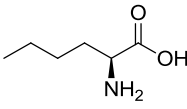
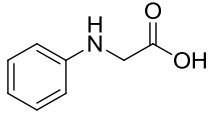
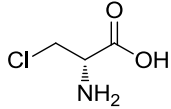
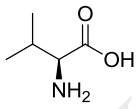
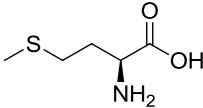
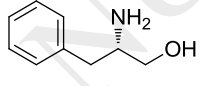
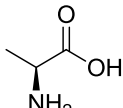
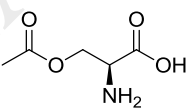


Scheme 2. ODH type catalysed reactions.

Other examples have since been identified in the soil bacteria, *Agrobacterium tumefaciens* (Heidekamp et al., 1983) and *Arthrobacter sp.* strain 1C (Asano et al., 1989; Dairi and Asano, 1995). Asano *et al.* assessed the substrate specificity of ODH using various amines against pyruvate as the α -keto acid. L-Methionine proved to be the favoured substrate followed closely by L-isoleucine, L-valine, L-phenylalanine, L-leucine, L-alanine, L-threonine, L-cysteine, L-serine, glycine and L-asparagine. A few other amino acids such as β -alanine or L-histidine were not tolerated and no product was observed (Asano et al., 1989). The same group also examined the preparative potential of the ODH from *Arthrobacter sp.* strain 1C and found it to give a different substrate selectivity pattern (Kato et al., 1996). The enzyme was cloned and recombinantly expressed in *E. coli* and shown to accept a wide range of amino acids as amine donors on reaction with pyruvic acid (Table 1). Interestingly, (*S*)-phenylalaninol (26), a non-zwitterionic amine, was tolerated as the amine donor and to the best of our knowledge, is the only example of a non-amino acid amine donor accepted by a wild-type enzyme of this class. Glyoxylate and 2-ketobutyrate were also accepted when reacted with L-isoleucine and L-valine, suggesting that the substrate scope could be extended. The reductive amination of this ODH proceeds with high D-(*R*)-stereoselectivity (>99%) that is common to many, but not all enzymes of this class. This is in contrast with the strict L-(*S*)-selectivity of other enzymes capable of *N*-alkylating α -amino acids.

Table 1. Selected substrate specificity of D-(*R*)-selective opine dehydrogenase from *Arthrobacter sp.* strain 1C in reaction with pyruvate (16).



Amine donor	Relative activity	Amine donor	Relative activity
	100		21
17		18	
	84		0.7
19		20	
	72		4
21		22	
	53		22
23		24	
	24		0.6
25		26	
	16		36
27		28	

ODHs are usually highly substrate specific, as demonstrated by the ODH from *Pecten maximus* which accepts pyruvic acid (16) and arginine as substrates (Smits et al., 2008). Other amino acid donors such as canavanine (25%), cysteine (1.2%), L-alanine (<1%), ornithine (<1%) and norvaline (<1%), display reduced or negligible activity. Another example is the OcdH from *Mytilus galloprovincialis*, which accepts alanine with approximately 10-fold less activity than arginine (88% versus 9%) (Vazquez-Dorado et al., 2011).

In invertebrates there are many types of ODH that have been identified such as alanopine dehydrogenases (EC 1.5.1.17; AIDH) (Storey, 1983); β -alanopine dehydrogenases (EC 1.5.1.26; β -AIDH) (Kan-no et al., 1999; Sato et al., 1987); strombine dehydrogenases (EC 1.5.1.22; StDH) (Dando, 1981) and tauropine dehydrogenases (EC 1.5.1.23; TaDH) (Gaede, 1986). It should be noted that these classifications are based on their preferred substrate specificity, and there is cross reactivity between ODH classes as many of these enzymes accept substrates of other classes, but with lower activities. This can be seen from the work of Sato *et al.* who tested a variety of ODHs from different marine

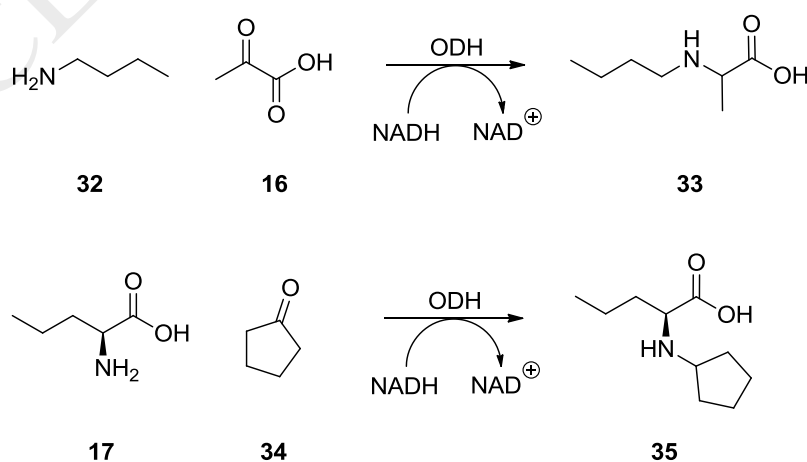
organisms towards various amino acid donors and demonstrated that most display a range of activities (Sato et al., 1993). AIDHs, for example, primarily operate on alanine. However Storey et al. tested the substrate specificity of AIDH from *Aphrodite aculeata* and found that several other amino acids were accepted, including glycine, serine and threonine, and a relatively broad range of keto acids (Table 2) with varying efficiencies (Storey, 1983).

Table 2. Selected keto acids specificity of AIDH from *Aphrodite aculeata* in reaction with L-alanine.

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Keto acid	V _{MAX}	Amine donor	V _{MAX}
 16	100	 29	21
 30	26	 31	107

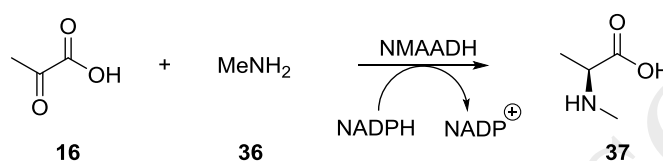
The value of these enzymes came to the attention of Codexis Inc. as a potential imine reduction platform if the substrate scope could be broadened to include other ketones and amines. They performed extensive engineering on *Anthrobacter sp.* opine dehydrogenase to expand the accepted substrate scope and identified mutants capable to tolerating substrates such as butylamine, cyclopentanone and an expended range of keto acids (Scheme 3) (Chen et al., 2013). However, these findings have not been disclosed outside of a patent and many details, such as the level of activity, enantioselectivity and exact sequences of the enzyme mutants, remain unclear.



Scheme 3. Examples of reactions carried out by evolved opine dehydrogenases.

2.2.2. *N*-Methyl amino acid dehydrogenases

N-Methyl amino acid dehydrogenases were first discovered in 1970 by Kung and Wagner (Kung and Wagner, 1970). They reported the formation of *N*-methylalanine when cell free extracts of *Pseudomonas* MS were treated with methylamine. In 1975, the same group managed to purify and characterise the enzyme, which they named *N*-methyl amino acid dehydrogenase for its ability to catalyse the reductive amination of pyruvate and methylamine (Scheme 4) (Lin and Wagner, 1975). *N*-Methyl amino acid dehydrogenases (NMAADHs, EC 1.5.1.1 and EC 1.5.1.21) are also known as Δ^1 -piperideine-2-carboxylate/ Δ^1 -pyrroline-2-carboxylate reductases (dPkAs), Pip2CRs or Pyr2CRs.

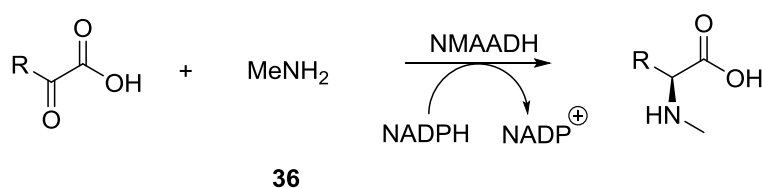
Scheme 4. NMAADHs from *Pseudomonas* MS producing *N*-methylalanine.

Substrate and cofactor specificity were also explored to determine whether other keto acids or amine sources could be accepted by the enzyme. When NADPH was substituted with NADH, conversions dropped from 100% to 4% for the reaction of pyruvate with methylamine. Other keto acids such as oxaloacetate and α -ketobutyrate were tolerated with diminished efficiency (64% and 14% respectively, relative to pyruvate) in the reaction with methylamine, whilst glyoxalate, hydroxypyruvate and α -ketoglutarate were not tolerated. Ethylamine and dimethylamine were not tolerated as substrates in the presence of pyruvate and crucially, little activity was observed towards ammonia.

Mihara et al. later found that the NMAADH from *Pseudomonas putida* ATCC12633 is also capable of performing the reductive amination between a number of additional α -keto acids and methylamine (Mihara et al., 2005). After it was cloned and expressed in *E. coli*, the enzyme displayed moderate activity towards fluorinated, aromatic and aliphatic pyruvate derivatives, but hydroxy and branched chain derivatives such as α -oxoisovalerate (42) were inert, with pyruvate being the best keto acid tested (Table 3).

Table 3. Reductive amination of some pyruvates with methylamine (36) with *Pseudomonas putida*

NMAADH.



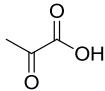
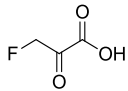
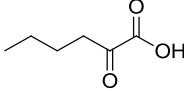
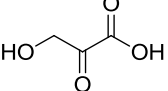
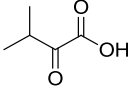
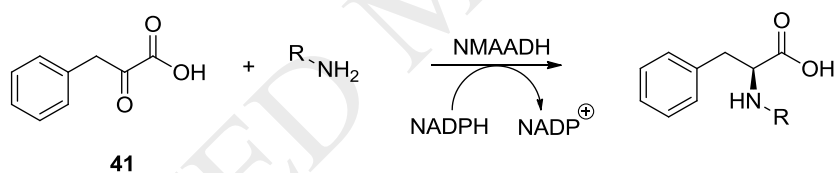


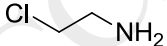
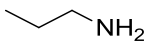

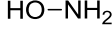

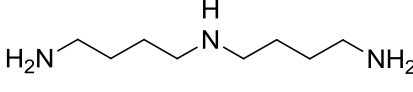
Keto acid	Relative activity	Keto acid	Relative activity
	100		27
16		38	
	52		6
39		40	
0	30		0
41		42	

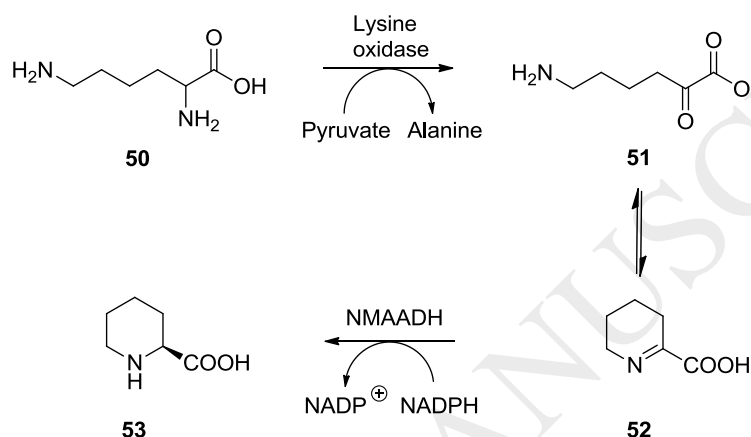
Table 4 shows the relative activity of *Pseudomonas putida* NMAADH towards the reductive amination of phenylpyruvate (41) with a range of amines, with methylamine (36) being the most effective by a large margin (Mihara et al., 2005). An increase of one carbon to (43) gives a large decrease in activity. As the chain length increases the activity continues to decrease, however the enzyme does tolerate large amines (such as spermidine (49)) to a small degree. Interestingly, this enzyme is inactive with ammonia and therefore does not form primary amines.

Table 4. The relative activity of *Pseudomonas putida* NMAADH.



Amine	Relative activity	Amine	Relative activity
	100		4.4
36		43	
	0.74		0.16
44		45	
	0.12		<0.06
46		47	
	0		<0.06
48		49	

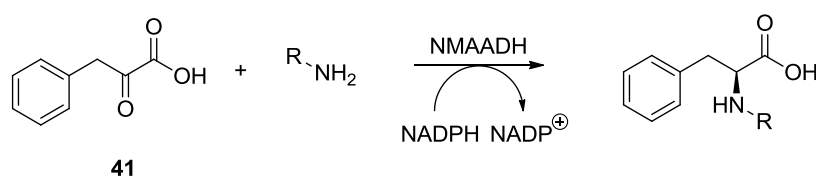
In 2004, the Esaki group demonstrated the synthetic utility of this enzyme by successfully producing *N*-methyl-L-phenylalanine on a 1.6 g scale with good yield and high enantiomeric excess by using NMAADH from *Pseudomonas putida* and glucose dehydrogenase from *Bacillus subtilis* (Muramatsu et al., 2004). Further studies from the same group have shown that NMAADHs can also accept a wide range of cyclic imino acids such as Δ^1 -piperidine-2-carboxylate and Δ^1 -pyrroline-2-carboxylate (Yasuda et al., 2006). Cyclic imino acids were produced spontaneously from their corresponding α -keto- ω -amino acids which, in turn, produced from their corresponding α,ω -diamino acids using amino acid oxidases. One of their best examples is highlighted in Scheme 5 where (*S*)-(**53**) was obtained in high yield tandem use of lysine oxidase and NMAADH.

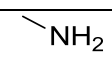
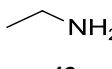
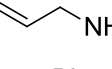
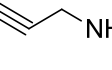
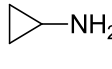


Scheme 5. Lysine degradation pathway steps incorporating NMAADHs.

Further studies have shown that NMAADHs from *Pseudomonas putida* can reduce a number of 2,3,4,5-tetrahydropyridine-6-carboxylate compounds containing oxygen or sulphur within the ring. (Esaki et al., 2008) Δ^1 -Piperidine-2-carboxylate and Δ^1 -pyrroline-2-carboxylate reductases have been also demonstrated to be involved in the catabolism of D-lysine and D-proline (Muramatsu et al., 2005). Recently, researchers from GSK have demonstrated that both keto acid and amine substrate scope can be further expanded with excellent enantioselectivities by using NMAADHs from *Pseudomonas putida*, *P. syringae* and *P. fluorescens* (Table 5) (Hyslop et al., 2018).

Table 5. Example of NMAADH catalysed reductive amination.



Amine	Conversions of NMAADHs		
	<i>Pseudomonas putida</i>	<i>Pseudomonas syringae</i>	<i>Pseudomonas fluorescens</i>
 36	97	98	98
 43	16	6	5
 54	12	6	5
 55	92	52	58
 56	59	27	29

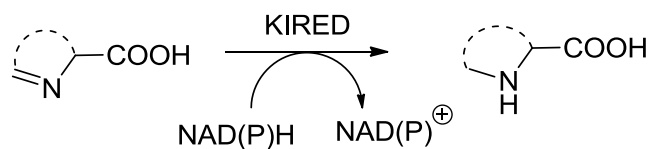
2.2.3. Ketimine reductases

Ketimine reductases (KIREDS, EC 1.5.1.25) are another class of enzyme that can carry out the reductive amination of imino acids. They were first discovered in mammalian tissues such as rat kidney, brain, liver, testes, heart, skeletal muscle and spleen (Meister, 1962; Meister et al., 1957) and rabbit liver (Meister and Buckley, 1957) and have been shown to catalyse the conversion of Δ^1 -piperidine-2-carboxylate and Δ^1 -pyrroline-2-carboxylate to their corresponding saturated cyclic amino acids (Table 6, Entries 1-2). Even though KIREDS perform the same natural reaction as NMAADHs, they are structurally distinct (Hyslop et al., 2018). Plant extracts from *Pisum sativum* and *Phaseolus radiatus* also catalyse the conversion of Pyr2C (52) to pipercolic acid (53), but detailed studies of the plant tissues were not performed. The activity of the plant extracts was found to be of the same order of magnitude as that observed with rat liver (Meister et al., 1957).

These enzymes, later found to also reside in pig kidney (Nardini et al., 1988a) and in brain μ -Crystallin (CRYM) (Hallen et al., 2011), have also been shown to reduce a variety of naturally occurring sulphur containing cyclic imino acids (Table 6). These compounds are found in varying concentrations within the human brain, although the relative activities of KIREDS towards these sulfur containing compounds was not specified (Hallen et al., 2015a).

Interestingly, both NADH and NADPH appear to be accepted equally in the case of pig kidney KIREDS and cerebral l-crystallin/KIREDS, but NADPH is the preferred cofactor for cerebellum KIREDS. All KIREDS display a strict enantio-preference for the formation of L-alkyl- α -amino acids.

Table 6. KIREDS substrate scope.

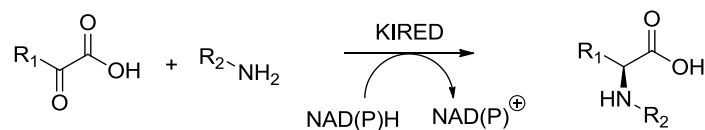


Substrate	Enzyme Source	Product	Reference
 52	Mammalian tissues	 53	(Meister and Buckley, 1957) (Meister et al., 1957) (Meister, 1962)
	Pig kidney		(Nardini et al., 1988a)
	Bovine Cerebellum		(Nardini et al., 1988b)
 54	Mammalian tissues	 55	(Meister and Buckley, 1957) (Meister et al., 1957) (Meister, 1962)
 56	CRYM/ Lamb forebrain	 57	(Hallen et al., 2011)
	Pig kidney		(Nardini et al., 1988a)
	Bovine Cerebellum		(Nardini et al., 1988b)
 58	Pig kidney	 59	(Nardini et al., 1988a)
	Bovine Cerebellum		(Nardini et al., 1988b)
 60	Pig kidney	 61	(Nardini et al., 1988a)
	Bovine Cerebellum		(Nardini et al., 1988b)
 62	Cerebellum	 63	(Hallen et al., 2014)

Hallen et al. further extended the substrate scope of the KIREdS by demonstrating that human ketimine reductase/CRYM, recombinantly expressed in *Escherichia coli* and purified, could catalyse the synthesis of four *N*-functionalised L-alkyl- α -amino acids (Table 7) (Hallen et al., 2015b). The relative

rates quoted in Table 7 are shown in comparison to piperidine-2-carboxylate and as can be seen, the rates for the acyclic keto acids are far lower, but still significant.

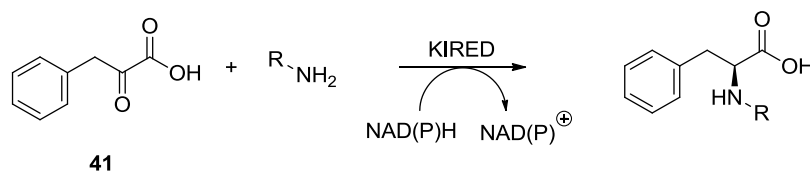
Table 7. Conversions of *N*-Functionalised amino acids synthesised using KIRED.



Ketone	Amine	Relative rate
 16	 36	25
 16	 43	24
 64	 36	16
 41	 36	12

The biocatalytic utility of KIREDs has recently been further demonstrated by Hyslop et al. (2018). Three recombinant KIREDs, which were cloned and expressed well in *E. coli*, were used to prepare a variety of phenylalanine derivatives on reaction with amines such as methylamine, propargylamine and benzylamine with pyruvate (Table 8). Conversions ranged from 32-81% in the case of methyl amine and were low to moderate when propargyl amine was used as amine donor (19-26%). Cyclopropyl amine was also accepted in good conversions (up to 79%) at 10 mM keto acid concentration.

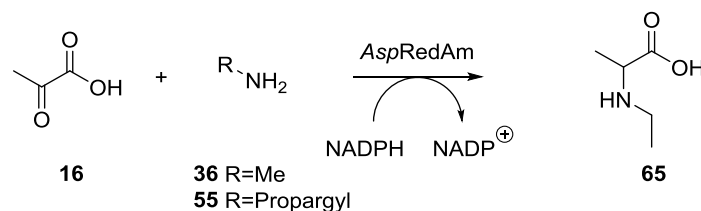
Table 8. Enzymatic reductive amination conversions for phenylpyruvic acid (41) catalysed by KIREDs.



Amine	Relative activity		
	<i>Rattus norvegicus</i>	<i>Homo sapiens</i>	<i>Bos taurus</i>
NH ₂ 36	51	81	32
NH ₂ 43	5	9	2
NH ₂ 54	16	30	10
NH ₂ 55	19	24	26
NH ₂ 56	43	79	29

2.2.4. Imine reductases

Imine reductases carry out the reduction of cyclic imines but have also been exploited in reductive amination reactions (Aleku et al., 2017; Cosgrove et al., 2018; Roiban et al.; Roth et al.; Velikogne et al., 2018). Despite the number of diverse IREDs reported, to the best of our knowledge, there is only one example demonstrating activity towards an α -keto acid. Aleku *et al.* screened the AspRedAm towards multiple substrates including pyruvate (16), in the presence of methylamine and propargylamine (Scheme 6) (Aleku et al., 2017). However, in the presence of a large excess of the amine donor (>4 equivalents) only modest activities were observed of 3% for methylamine (36) and 4% for propargylamine (55).



Scheme 6. IRED catalysed reductive amination of pyruvate (16) with ethylamine (43).

As research into the IRED field continues to expand rapidly, with many examples of new enzymes and evolution projects, it is likely that more effective examples will be identified in the coming years.

2.3. Other enzyme classes

There are a few other examples of some specific enzymes able of forming *N*-functionalized amino acids. Pyrroline-5-carboxylate reductases (P5CRs, EC 1.5.1.2) are a class of enzymes involved in the biosynthesis of proline, which is formed following reduction of pyrroline-5-carboxylate (Singh et al., 2013). They are perhaps the least characterised of the oxido-reductases described in this chapter in spite of their substrate similarity to that of NMAADHs and KIREDs (Yura, 1959). While several P5CR examples have been identified, none have been used in preparative synthesis. The P5CR from *Neurospora crassa* has been recently screened towards a panel of substrates and accepts methylamine, ethylamine, allylamine and propargylamine with conversions varying from 2% up to 82% (Hyslop et al., 2018). While only one enzyme has been demonstrated to have this activity, there is potential to further explore this enzyme class for amino acid synthesis activity.

Ammonia lyases (AL, EC 4.3.1) are enzymes commonly found in plants and fungi, which carry out either the deamination of amino acids to α,β -unsaturated carboxylic acids and ammonia or the reverse amination process (Lovelock and Turner, 2014). These enzymes accept ammonia as their amine source, but there are very few examples of any other amines being accepted. Viergutz et al. demonstrated that a wild type PAL (from *P. crispum*) was able to deaminate *N*-methyl phenylalanine (62), but did not carry out the reverse reaction at a detectable rate (Viergutz et al., 2003). Additionally, this PAL did not accept 4-nitro-*N*-methyl phenylalanine or 4-nitro-*N,N*-dimethyl phenylalanine in either direction.

Preparation of *N*-substituted aspartic acids via enantiospecific conjugate addition of *N*-nucleophiles to fumaric acids has been successfully demonstrated by Gani et al. using methylaspartase (Saeed Gulzar et al., 1997). Aspartate ammonia lyase is another enzyme type which proved to be an excellent catalyst for the synthesis of *N*-substituted aspartic acids. In this context Weiner et al. demonstrated the enantioselective AspB-catalyzed addition of methylamine to fumarate (Weiner et al., 2008).

3. Conclusions

N-functionalised amino acids are versatile building blocks for the pharmaceutical and fine chemical industries and are particularly important starting materials for peptide drugs. There are now a variety of preparative biocatalytic options to access these materials in enantiopure form, therefore offering a cleaner and greener alternative to chemical synthetic methods which often employ heavy metal catalysts, toxic reagents, environmentally unfriendly solvents and the extensive use of protecting groups. We anticipate they will supplant current chemical methods as improved, engineered enzyme variants for these transformations become available.

4. Acknowledgements

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