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An Improved Racemase/Acylase Biotransformation for the Preparation of Enantiomerically Pure Amino Acids

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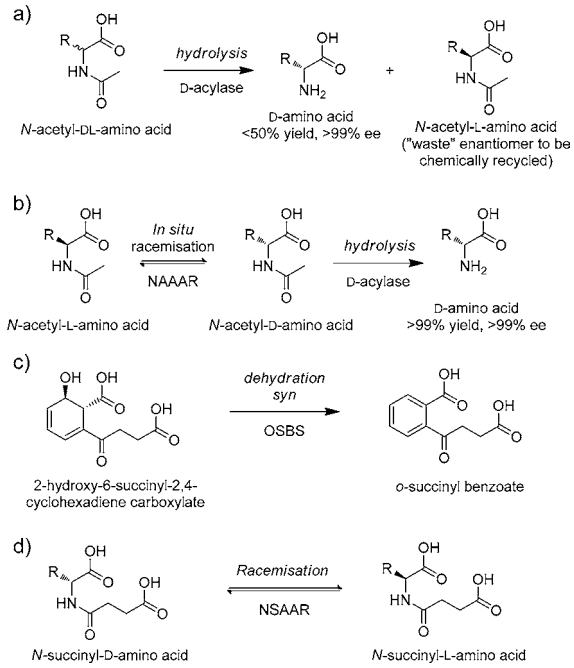
Supporting Information

ABSTRACT: Using directed evolution, a variant *N*-acetyl amino acid racemase (NAAAR G291D/F323Y) has been developed with up to 6-fold higher activity than the wild-type on a range of *N*-acetylated amino acids. The variant has been coupled with an enantiospecific acylase to give a preparative scale dynamic kinetic resolution which allows 98% conversion of *N*-acetyl-DL-allylglycine into D-allylglycine in 18 h at high substrate concentrations (50 g L^{-1}). This is the first example of NAAAR operating under conditions which would allow it to be successfully used on an industrial scale for the production of enantiomerically pure α-amino acids. X-ray crystal analysis of the improved NAAAR variant allowed a comparison with the wild-type enzyme. We postulate that a network of novel interactions that result from the introduction of the two side chains is the source of improved catalytic performance.

Kinetic resolutions (KRs) that employ stereoselective acylases are commonly used for the production of both L- and D-α-amino acids from their *N*-acetylated-DL starting materials.[1−4] However, a drawback in this process is the need for repeated chemocatalytic racemisation steps of the non-desired enantiomer to achieve yields >50% (Scheme 1a). These racemisation steps, if performed at all, are typically implemented under harsh chemical conditions resulting in extra cost and waste. A solution to this problem would be a dynamic kinetic resolution (DKR), driven by an *in situ* racemisation step that would allow yields to approach 100% (Scheme 1b). A cheap and "green" catalyst for this racemisation would be an enzyme, such as *N*-acyl amino acid racemase (NAAAR) from the actinobacterium *Amycolatopsis* sp. Ts-1−60. This enzyme has many desirable features in that it requires no organic cofactor, is active at high temperatures, accepts a wide range of amino acid substrates, and suffers no substrate or product inhibition up to 300 mM. Unfortunately, the activity of this NAAAR with the desired *N*-acetyl substrates is too low for commercial use. To amend this, we have used directed evolution to generate NAAAR variants with increased racemase activity toward an expanded range of *N*-acetyl amino acids. Directed evolution has proven to be a highly versatile and successful tool for protein engineering.[5−8] However, finding improved enzymes is often technically difficult or time consuming due to the large number of variants that must be screened. In the case of NAAAR this is especially true; the substrate and product are enantiomers, which makes assay development difficult. To overcome this, we have generated an enantioselective genetic selection system that allows for high-throughput screening of mutagenic NAAAR libraries.

Scheme 1. (a) D-Acylase-Based Kinetic Resolution of a *N*-Acetyl-DL-amino Acid to Yield an Enantiopure D-Amino Acid; (b) NAAAR/Acylase Coupled Dynamic Kinetic Resolution; (c) Natural Reaction of the NAAAR as an OSBS in the Menaquinone Pathway; (d) Enzyme Can Also Act as an NSAAR



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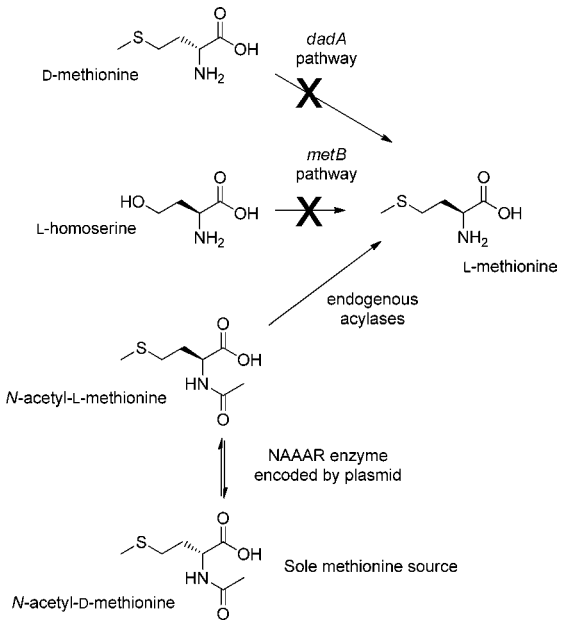
As well as *Amycolatopsis* species, NAAAR activity has also been detected in *Streptomyces atratus* Y-53 and in several thermophiles.[9−14] In these bacteria, NAAAR is believed to act as a bifunctional enzyme, as an *o*-succinyl benzoate synthase (OSBS) that catalyzes the formation of *o*-succinylbenzoate (a precursor of menaquinone) from 2-hydroxy-6-succinyl-2,4-cyclohexadiene carboxylate (Scheme 1c)[15] as well as an *N*-succinyl-amino acid racemase (NSAAR) involved in D-amino acid detoxification (Scheme 1d).[16] NAAAR has been well studied as a member of the enolase superfamily, and mechanistic analysis has shown that the NAAAR reaction proceeds via a divalent cation-dependent, enolate-based proton transfer catalyzed by two lysine residues.[17] The racemization of *N*-succinyl amino acids (and the OSBS reaction) is known to be ∼20-fold faster than with the equivalent *N*-acetylated substrates. As the present acylase process for the production of enantiopure α-amino acids requires *N*-acetylated substrates, high activity toward these derivatives is essential. Our aim was to isolate a NAAAR variant with sufficiently high activity, such that the current acylase KR process could be run as an efficient, coupled NAAAR/acylase DKR. We achieved this by NAAAR gene mutagenesis followed by genetic selection in an engineered *Escherichia coli* host.

To efficiently screen mutagenic *Amycolatopsis* sp. Ts-1−60 NAAAR libraries, a selection method was required that linked the rate of racemisation of an *N*-acetyl amino acid to the viability of an *E. coli* host. This has been achieved by disabling the natural *E. coli* L-methionine biosynthetic pathway, and at the same time removing a D-amino acid racemization pathway (Scheme 2). Conversion of L-homoserine to L-methionine was abolished by deletion of the second enzyme in the L-methionine biosynthetic pathway, cystathionine γ-synthase (metB), while D-amino acid racemization was prevented by deletion of a nonspecific D-amino acid dehydrogenase (dadA). DadA combines with an aminotransferase to racemise a variety of amino acids via their prochiral keto acid allowing toxic D-amino acids to act as a source of useful L-amino acids. As a result of these deletions, when grown in minimal media containing *N*-acetyl-D-methionine as the sole methionine source, the selection host is now dependent on recombinant NAAAR activity to racemise this to *N*-acetyl-L-methionine. An endogenous hydrolase then provides the source of L-methionine from the *N*-acetylated substrate. Using the λ red-mediated gene replacement/deletion method, dadA was deleted, and metB replaced with a chloramphenicol resistance gene.[18] This double-knock out host, named SET21, is now completely unable to synthesize L-methionine or racemise D-methionine. Importantly, the SET21 host is viable on *N*-acetyl-L-methionine allowing for a NAAAR-dependent screening method shown in Scheme 2. The ability of NAAAR to complement the SET21 strain and produce viable colonies was confirmed by transformation of SET21 with a plasmid expressing NAAAR wild-type (WT) and selection on minimal media supplemented with *N*-acetyl-D-methionine as the sole methionine source (see Supporting Information, SI).

Scheme 2. Disabled L-Methionine Biosynthesis in *E. coli* SET21 Host and NAAAR-Dependent Growth on *N*-Acetyl-D-methionine



Using the SET21 selection host, mutagenic libraries of the NAAAR gene were screened, and the best variant used as the template for the next round of mutagenesis/selection. Variants were generated using XL1-Red (>10^7 variants), error prone PCR (>10^5 variants), and saturation mutagenesis (>10^2 variants). Active variants were selected by SET21 colony size when grown on minimal media supplemented with *N*-acetyl-D-methionine or by high-throughput HPLC screening (see SI). Selection of libraries generated by XL1-Red mutagenesis led to the isolation of the G291E mutation, which was subsequently improved upon by saturation mutagenesis to generate G291D. Error prone PCR on this clone generated NAAAR G291D F323Y, which could not be improved upon after saturation mutagenesis at residue 323 and a further round of error prone PCR. Saturation mutagenesis of other residues (D316 and S135) within the active site of NAAAR G291D F323Y failed to generate improved variants. The NAAAR activity of each variant was determined by expression and purification of recombinant protein followed by kinetic analysis with a range of amino acids. The rate of racemisation was monitored with chiral HPLC, and the activities of the best variant from each round of selection are shown in Table 1. As well as testing both enantiomers of methionine (used in the plate selection assay), we also chose two synthetically useful amino acid precursors to probe the versatility of the new variant: D-(4-fluoro)phenyl glycine and D-allylglycine since they are used in the preparation of various drugs. Gratifyingly, the NAAAR G291D F323Y variant displayed the most improved activity with each of the substrates tested, showing up to 6-fold improved k_{cat} values over WT. The kinetic data would suggest that the mutations are directly affecting turnover and not substrate binding, as no large changes in K_m values were observed with each amino acid.

To measure the improvement in the DKR of *N*-acetyl-DL-amino acids with these variant NAAARs, small scale resolutions were performed using whole cell biocatalysts expressing an L-acylase and one of NAAAR WT, G291E, G291D, or G291D F323Y. Combinations of the acylase and NAAAR hosts were mixed, and the resolution of *N*-acetyl-DL-methionine to L-methionine monitored over 5 h by chiral HPLC (Figure 1). As expected, with no NAAAR present, *N*-acetyl-D-methionine showed no conversion to free L-methionine (data not shown). When BL21(DE3) cells expressing NAAAR WT were used, *N*-acetyl-D-methionine was now a substrate, via racemisation, for the L-specific acylase, however the rate of racemization (k_{rac}) was

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Table 1. Kinetic Parameters of NAAAR Variants with Various *N*-Acetylated Amino Acids

	N-acetylated amino acid substrate							
	L-methionine		D-methionine		D-(4-fluoro)phenylglycine		D-allylglycine	
variant	k_{cat} (s^{-1})	K_m (mM)	k_{cat} (s^{-1})	K_m (mM)	k_{cat} (s^{-1})	K_m (mM)	k_{cat} (s^{-1})	K_m[a] (mM)
WT	20	18	14	40	41	35	97	nd
G291E	48	40	27	40	103	35	125	nd
G291D	80	40	54	40	125	35	153	nd
G291D F323Y	95	40	67	50	272	40	232	nd

[a] K_m could not be calculated with *N*-acetyl-allylglycine as no saturation of activity was observed up to tested levels of 300 mM.

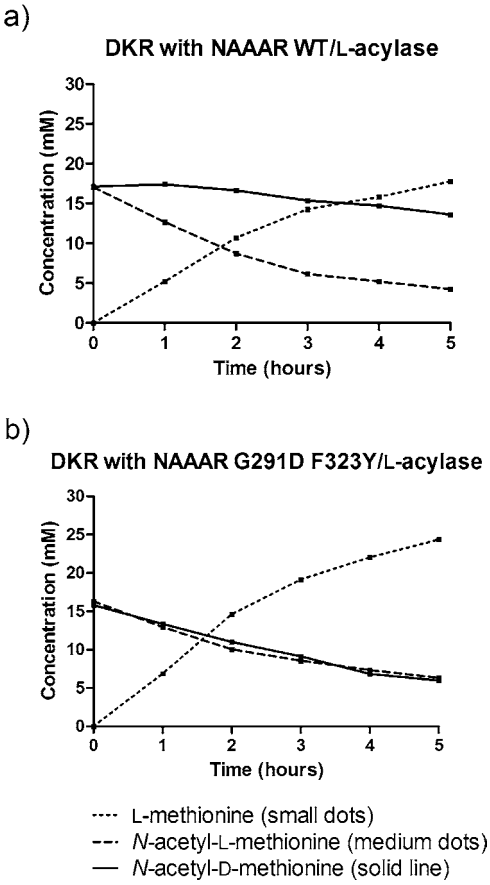

Figure 1. DKR of *N*-acetyl-DL-methionine by the coupled NAAAR/L-acylase process with (a) NAAAR WT and (b) NAAAR G291D F323Y. The decrease in *N*-acetyl-D-methionine concentration gives a measure of racemase activity (k_{rac}), and the drop in *N*-acetyl-L-methionine gives a measure of acylase activity (k_{hyd}). No major increase in L-methionine yield is observed as the reaction has not gone to completion and is limited by L-acylase turnover.

clearly slower than the rate of hydrolysis (k_{hyd}) (Figure 1a). Similar results, with improved k_{rac} values were observed for both G291 variants, however k_{rac} was still slower than k_{hyd} (data not shown). In 5 h the WT NAAAR/L-acylase DKR turned over ∼50% of the initial racemic methionine substrate to L-methionine as expected (Figure 1a, dotted line). Encouragingly, in the NAAAR G291D F323Y/L-acylase DKR, both enantiomers of *N*-acetyl-methionine were being consumed at the same rate, i.e., $k_{rac} \geq k_{hyd}$ (Figure 1b). This resulted in ∼80% conversion to L-methionine. Left longer the reaction would approach 100%, but the diminishing substrate concentration and limiting L-acylase activity slow the reaction down. The NAAAR G291D F323Y/L-acylase combination runs as an effective DKR since the ee of the L-methionine produced was >99% (by chiral HPLC).

The performance of NAAAR G291D F323Y under process-like conditions was further investigated with a DKR of *N*-acetyl-DL-allylglycine using a partially purified NAAAR G291D F323Y biocatalyst generated from an *E. coli* fermentation (see SI). A coupled NAAAR G291D F323Y/D-acylase process was used to resolve 1g of *N*-acetyl-DL-allylglycine to D-allylglycine with ∼98% yield (see Figure S3) (89% isolated product) in 18 h. The equivalent D-acylase kinetic resolution would give only a theoretical maximum of 50% conversion. The low enzyme loading required and excellent yields suggest these processes would be economical at scale. Moreover, we show the versatility of the DKR since the NAAAR variant works well with both D- and L-acylase combinations.

Analysis of the WT NAAAR:*N*-acetyl-methionine structure (PDB: 1SJA) revealed that both G291 and F323 are found within the *N*-acyl binding pocket, ∼5−6 Å from the substrate acyl group.[19] To gain an insight into the molecular basis for the improvement in catalysis, the X-ray crystal structure of NAAAR G291D F323Y (PDB: 4A6G) was determined. A data set to a resolution of 2.71 Å was collected, and the structure solved using 1SJA as a model (see SI). Two novel interactions were observed arising from the G291D and F323Y side chains (Figure 2a). These are a salt bridge between the carboxylate of D291 and the guanidinium of R299 (2.7 Å) and an H-bonding interaction between the carboxylate of D291 and the hydroxyl of Y323 (2.8 Å). The resolution of the structure prevented identification of definitive changes in protein−ligand interactions; however, the ligand was slightly displaced compared to the published WT structure, suggesting that its binding environment had been altered.

The most interesting observation can be made when comparing the structures of NAAAR WT complexed with *N*-succinyl-methionine (PDB: 1SJC)[19] and the NAAAR G291D F323Y:*N*-acetyl-methionine complex. Here, the carboxylate of G291D can be seen to be mimicking the succinyl carboxylate which is not present on the acetyl group. In the NAAAR WT:*N*-succinyl methionine complex this interaction is made via two structural water molecules (Figure 2b). Since NAAAR WT is 10−20-fold more active with *N*-succinyl amino acids than the equivalent *N*-acetyl substrates, it appears this interaction is of key importance for efficient turnover. The interaction between the newly introduced side-chains and the absence of interactions between these and the substrate is complemented by kinetic data that suggest that D291 and Y323 have increased catalytic turnover but not affected ligand binding.

In summary, a variant NAAAR with increased activity toward synthetically useful *N*-acetyl amino acids has been evolved that shows promise as an industrial biocatalyst. When coupled with a stereoselective acylase, gram level resolutions with excellent

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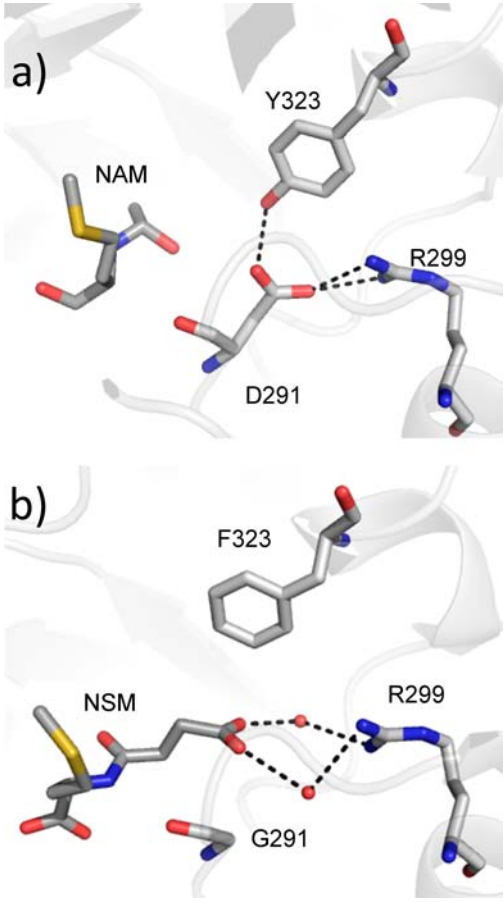

Figure 2. (a) New interactions observed in the NAAAR G291D F323Y acyl binding pocket, NAM = *N*-acetyl-methionine. (b) Interactions between succinyl carboxylate and R299 via two water molecules (red spheres), NSM = *N*-succinyl-methionine.

yields could be achieved within 18 h at 50 g L^{-1} substrate concentrations. Initial results would suggest these processes will be cost-effective at scale for the production of a range of both proteinogenic and nonproteinogenic α-amino acids.

■ ASSOCIATED CONTENT

Supporting Information

Descriptions of experimental procedures. This material is available free of charge *via* the Internet at http://pubs.acs.org.

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Notes

The authors declare the following competing financial interest(s): Due to the nature of the research four of the seven authors are employed by industrial companies and have commercial interests in the use of the enzymes in this report.

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