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Continuous Colorimetric Assay That Enables High-Throughput Screening of *N*-Acetylamino Acid Racemases

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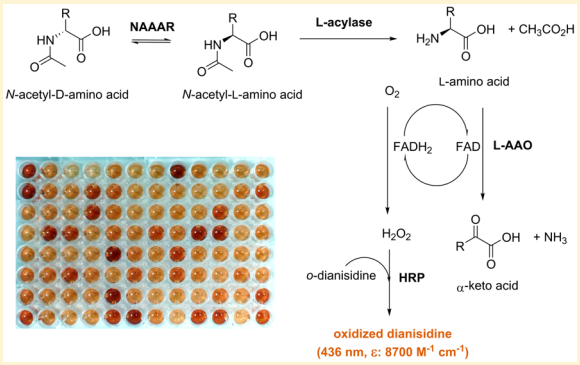
Supporting Information

ABSTRACT: *N*-Acetyl amino acid racemases (NAAARs) have demonstrated their potential in the enzymatic synthesis of chiral amino acids, molecules of significant biotechnology interest. In order to identify novel activities and to improve these enzymes by engineering approaches, suitable screening methods are necessary. Previous engineering of the NAAAR from *Amycolatopsis* Ts-1-60 was achieved by relying on an *in vivo* selection system that linked the viability of an *E. coli* L-methionine auxotroph to the activity of the improved enzyme. However, this assay was only suitable for the screening of *N*-acetyl-D-methionine, therefore limiting the potential to evolve this enzyme toward other natural or non-natural acetylated amino acids. Here, we report the optimization and application of a spectrophotometric microtiter-plate-based assay for NAAAR. The assay is based on the detection of the amino acid reaction product formed by hydrolysis of the *N*-acylated substrate by an L-amino acid acylase and its subsequent oxidation by an FAD-dependent L-amino acid oxidase (L-AAO). Cofactor recycling of the L-AAO leads to the formation of hydrogen peroxide which is easily monitored using horseradish peroxidase (HRP) and *o*-dianisidine. This method allowed for the determination of the kinetic parameters of NAAAR and led to the identification of *N*-acetyl-D-naphthylalanine as a novel NAAAR substrate. This robust method is also suitable for the high-throughput screening of NAAAR mutant gene libraries directly from cell lysates.



Natural and unnatural α-amino acids have significant industrial interest as they have been commonly used as chiral building blocks for drugs and intermediates.[1−7] Hence, development of generally applicable, cost-effective and green technology for production of single enantiomeric unnatural amino acids is of significant industrial interest. Natural proteinogenic L-amino acids are readily available and inexpensive. In addition, racemic amino acids with a variety of unnatural side-chains are generally readily prepared synthetically. Therefore, efficient methods that convert L-amino acids to their D-enantiomeric forms or resolve racemic amino acid mixtures into either the L- or D-amino acids provide a simple, general, and economically viable route to the full range of enantiopure products. For example, processes that are based on hydantoinase/carbamoylase have been described and are used industrially for the production of D-amino acids.[1] A coupling system involving an *N*-acetylamino acid racemase (NAAAR) and an enantio-specific acylase (Scheme 1) allows the preparation of pure L- or D-amino acids from readily synthesized racemic *N*-acetylated amino acids. This matched acylase/racemase combination provides an ideal "dynamic kinetic resolution (DKR)" which allows the preparation of products in high yield and enantiomeric excess.[8−12]

Previous work selected *Amycolatopsis* *N*-acetyl amino acid racemase (NAAAR) as a good biocatalyst (UniProt sequence Q44244) with many desirable features;[8,13,14] it requires only a divalent metal cation for activity, is active at high temperatures (60 °C), accepts a wide range of amino acid substrates, and suffers no substrate or product inhibition up to 300 mM. Unfortunately, the activity of the wild type NAAAR with the desired hydrophobic *N*-acetylated substrates (e.g., phenylalanine and naphthylalanine) was too low for industrial use.

A current goal is to improve and expand the application of natural enzymes as biocatalysts using protein engineering tools such as random mutagenesis (epPCR) or saturation mutagenesis in a semirational, directed evolution approach.[15−20] This last strategy is aided by detailed knowledge of the substrate profile of the target enzyme, as well as an understanding of the enzyme mechanism and identification of the active site residues revealed by the crystal structure.[14] These combined techniques were applied previously to the NAAAR enzyme and led to the isolation of a double mutant (NAAAR G291D/F323Y) with up to 6-fold improvement of activity toward several *N*-acetylated amino acids. When the NAAAR G291D/F323Y mutant was combined with the L-acylase in a DKR, it allowed the preparation of pure L-allyl-glycine at 99%

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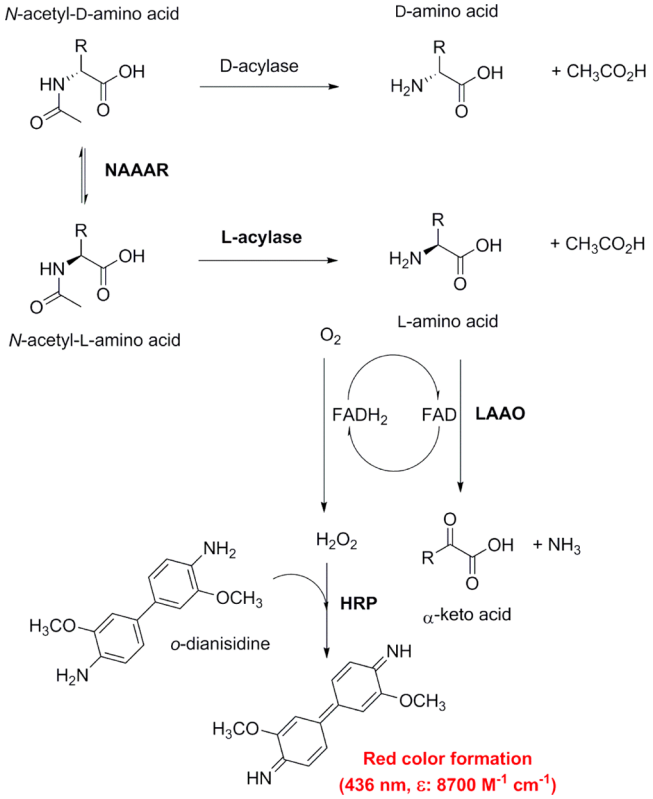
Scheme 1. Overview of the Coupled NAAAR/Acylase Dynamic Kinetic Resolution (DKR) and Description of the L-AAO/HRP-Dependent HTP Colorimetric Assay



e.e. from the racemic starting material. This NAAAR mutant was isolated using an *in vivo* selection assay constructed so as to ensure survival of an *E. coli* host strain only if it harbored the recombinant NAAAR.[21] For this method an L-methionine auxotrophic *E. coli* strain (SET21) was generated by making two specific gene knockouts (*dad*A and *met*B), and, as a result, when grown in L-methionine deficient media, the SET21 host was now dependent on plasmid-encoded NAAAR activity to convert the *N*-acetyl-D-methionine to *N*-acetyl-L-methionine which acts as a source of L-methionine.[14] This selection assay was useful for initial screening of NAAAR libraries but relied heavily on judgment calls on the size/growth of *E. coli* colonies and could only be tested using *N*-acetyl-D-methionine as a substrate. These limitations prohibit the potential evolution of NAAARs toward other non-natural and commercially valuable substrates. Similarly, a chiral HPLC method can be also used to quantitatively determine the activity of novel NAAAR mutants, but this method, although simple and accurate, does not allow the screening of the large number of variants typically produced in a directed evolution screening campaign.

Therefore, the evaluation of the activity of a library of enzymes variants toward a range of substrates requires a suitable method for high-throughput screening in microtiter plate format. This must be rapid, sensitive, reliable, and widely applicable. Here, we report the development of a new continuous colorimetric assay for the high-throughput screening of NAAAR. The assay is based on the production of free amino acids from their acetylated counterparts by linking NAAAR with an appropriate aminoacylase (Scheme 1). The oxidation of the resultant amino acid by an amino acid oxidase (L-AAO) can be linked to the production of hydrogen peroxide in a similar manner to the monoamine oxidase from *Aspergillus niger* (MAO-N).[22,23] The peroxide can be readily monitored by using horseradish peroxidase (HRP) to catalyze formation of a highly colored product (e.g., at 436 nm using the substrate *o*-dianisidine, Scheme 1).[24,25] The results obtained from kinetic measurements were verified by a chiral HPLC method. The colorimetric assay can be used with a range of different acetylated amino acids and allows the reliable determination of the specific activity and kinetic constants of NAAARs; it clearly differentiates between the wild-type NAAAR and the improved G291D/F323Y mutant. Also, since the aminoacylases and L-AAO accept a broad variety of natural and non-natural amino acids, this method is particularly suitable for exploring the substrate tolerance of NAAARs. Finally, the robustness and broad utility of the method allows the quantitative screening of mutant NAAAR libraries.

EXPERIMENTAL PROCEDURES

Chemicals. *N*-Acetyl-D-naphthylalanine was prepared by Dr. Reddy's Laboratories Ltd. (Cambridge, UK) by acylation of the free amino acid. *N*-Acetyl-D/L-methionine, D/L-methionine, *N*-acetyl-D-phenylalanine, *N*-acetyl-D-phenylglycine, *o*-dianisidine, magnesium chloride, and all other chemicals were obtained from Sigma-Aldrich unless otherwise stated.

Enzymes. The NAAAR gene (wild type and the G291D/F323Y double mutant), codon optimized for expression in *E. coli*, was cloned in pET26b, and recombinant NAAAR was prepared as described below. Recombinant L-acylase from *Thermococcus litoralis*[26] was prepared by Dr. Reddy's laboratories (specific activity: 39.7 U/mg). L-Amino acid oxidase (L-AAO, Cat. No. A5147) from *Crotalus atrox* and Horseradish peroxidase (HRP, Cat. No. P8125) were purchased from Sigma.

NAAAR Expression and Purification. An overnight culture of *E. coli* BL21(DE3) cells transformed with pET26b/NAAAR wild type or pET26b/NAAAR G291D/F323Y was used to inoculate 500 mL of TB broth (30 μg mL^{-1} kanamycin) and left to incubate at 37 °C with shaking until an OD_{600} of ~1.2 was reached. The cells were then induced with 0.4 mM IPTG at 20 °C for 16 h. Cells were harvested by centrifugation (4,000 rpm, 10 min) and resuspended immediately in 10 mL of 50 mM sodium phosphate (pH 8.0), 100 mM NaCl, and 1 complete EDTA-free mini-protease inhibitor tablet (Roche). This homogeneous solution was then sonicated on ice (30s on/30s off) for 10 min. The supernatant was then heated to 65 °C for 30 min to precipitate thermolabile proteins, clarified by centrifugation (12,000 rpm, 60 min, at 4 °C), and filtered (0.45 μm). Purification was carried out by anion exchange chromatography (HiPrep 16/10 FF Q column, GE Healthcare) and size exclusion chromatography (HR S300 column, GE Healthcare).

For plate screening, distinct separated transformed colonies were picked and transferred to individual wells in 48-well microplates containing 500 μL autoinduction medium with 100 μg/mL kanamycin, followed by 48 h incubation at 30 °C. Two ×48-well plates were then combined in one 96-deep well plate, and OD_{600} was normalized to an OD_{600} of 3 in all wells, followed by centrifugation to harvest the cells. Cell lysis was carried out by resuspending the cell pellets with a mixture of 100 mM sodium phosphate buffer pH 8.0, 25% BugBuster (primary amine-free, Merck) extraction reagent, and 5 U/mL benzonase (sigma), followed by incubation at room temperature for 1 h with shaking (200 rpm). The clarified cell-free

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extract was obtained after centrifugation (20 min, 4000 rpm). Autoinduction media: 10 g/L N-Z-amine, 5 g/L yeast extract, 1 mM $MgSO_4$, 1X NPS (20X NPS: 66 g/L $(NH_4)_2SO_4$, 136 g/L KH_2PO_4, 142 g/L Na_2HPO_4), 1X 5052 (50X 5052:250 g/L glycerol, 25 g/L glucose, 100 g/L α-lactose), 100 μg/mL kanamycin.

Colorimetric Assay for Detection of NAAAR Activity. Microtiter plate assay: NAAAR activity of 96-well plate cell extracts was measured continuously using a Biotek Synergy plate reader. Each well contained 100 μL cell lysate at an appropriate dilution and 100 μL assay mixture. Typical assay mixtures contained the following: 10−30 mM *N*-acetyl-D-amino acid, 5 mM $MgCl_2$, 79.4 U/mL L-acylase, 70 mU/mL L-AAO, 10 U/mL HRP, 0.1 mg/mL *o*-dianisidine in 50 mM sodium phosphate buffer pH 8.0. The increase in absorbance was monitored at 436 nm for 1 h, at 42 °C. Rates were determined from the slopes of the linear portion of each curve using an extinction coefficient for *o*-dianisidine of 8700 M^{-1} cm^{-1} and a path length of 0.58 cm for 200 μL reactions. Relative activity was calculated by subtracting the value obtained in the negative controls (cells transformed with empty plasmid) from each well.

Determination of the saturating amounts of coupling enzymes (L-acylase, L-AAO, and HRP) was carried out by adding increasing concentrations of each enzyme separately to the reaction. The best NAAAR concentration was also determined by lowering the NAAAR concentration until linearity was achieved.

To quantify the reproducibility and uncertainty of the screen, repeated measurements on single clones were carried out. Measuring the variability of the screen is important to determine a lower limit above which an observed improvement in a targeted property (e.g., enzyme activity) can be considered significant. The variability or dispersion of data was determined using the coefficient of variance [CV = (standard deviation/mean)*100%].[27] The CV should be as small as possible (<10%) to allow the detection of small improvements. The Z'-factor was also calculated to compare and evaluate the quality of the assay as described elsewhere.[28]

$$Z' = 1 - \frac{3\text{SD positive control} + 3\text{SD negative control}}{|\text{mean positive control} - \text{mean negative control}|}$$

HPLC based assay: This was similar to that previously reported[14,29] with the following modifications. For the HPLC method, reactions containing 0.2 mg/mL NAAAR, 79.4 U/mL L-acylase, 5 mM $MgCl_2$, and 10−30 mM *N*-acetyl-D-amino acids in 50 mM sodium phosphate buffer were incubated at 50 °C before being terminated at different times by diluting 40 fold in 0.025% triethylammonium acetate (TEAA):MeOH (50:50). Reactions were then analyzed by chiral HPLC using a Chirobiotic T column (Astec, chiral phase Teicoplanin, 5 μm, 250 mm × 4.6 mm) and the following isocratic conditions: mobile phase: 0.025% TEAA:MeOH (50:50, v/v), flow rate: 0.5 mL min^{-1}, detection λ: 210 nm, temperature: 25 °C, run time: 10 min. The column was calibrated with single enantiomers of the free amino acids and their *N*-acetyl derivatives. The conversion of *N*-acetyl-D-methionine to L-methionine was used to measure enzyme activity. Chromoleon software (Dionex) was used to measure the area under substrate and product peaks.

Kinetic Measurements. Kinetics parameters were determined using the microtiter plate assay at 50 °C and were calculated using the following conditions: 79.4 U/mL L-acylase, 5 mM $MgCl_2$, 70 mU/mL L-AAO, 10 U/mL HRP, 0.1 mg/mL *o*-dianisidine (in MeOH), various concentrations of *N*-acetyl-amino acids, 12.5 μg/mL NAAAR G291D/F323Y. By varying the concentration of the substrate, the obtained kinetic data were fitted using Graph Pad Prism software by nonlinear least-squares regression using the Michaelis−Menten equation. All k_{cat} values were defined as per NAAAR monomer. The concentration of protein was determined by measuring the absorbance at 280 nm. The extinction coefficient of NAAAR was calculated using Vector NTI software. Measurements were obtained in triplicate.

RESULTS AND DISCUSSION

General Description of the Enzyme-Coupled Assay. NAAAR and L-acylase together catalyze the conversion of *N*-acetylated-D-amino acids to L-amino acids. Rather than make a chromogenic derivative of the L-amino acid, our approach was to detect the product using a suitable, broad substrate coupling enzyme system (Scheme 1). We chose the flavin-dependent L-AAO/HRP pair in which the L-AAO initially oxidizes the free amino acid to its corresponding keto-acid.[30,31] Oxidation of the substrate by the flavin cofactor (FAD) generates $FADH_2$ which is then reoxidized by molecular oxygen, which itself is reduced to hydrogen peroxide (H_2O_2) by HRP. The resulting peroxide can be readily monitored by using *o*-dianisidine which reacts to form a colored product that absorbs at 436 nm (ε_{436} = 8700 M^{-1} cm^{-1}, Scheme 1).

Enzyme Linearity. To confirm the linearity of the reaction and make sure that the observed rates were not limited by the coupled reaction components, initial velocities were measured by varying the concentrations of both the NAAAR and the coupling enzymes. It is worth noting that we used double mutant NAAAR (G291D/F323Y) to optimize the assay since it has higher activity than the wild type. When the reaction was initiated by NAAAR addition, robust initial velocities were measured, and only low background rates were observed in the absence of enzyme or substrate (Supporting Information Figure S-1). The initial velocities were always calculated from the linear portions of the curves, after a lag phase of approximately 5 min. This lag phase is not unusual in enzyme-coupled reactions and is likely due to the required accumulation of free amino acids for the subsequent coupled reaction before a linear response is achieved. Figure 1 shows a linear relationship between the hydrogen peroxide production rate and NAAAR concentration using two different substrates, *N*-acetyl-D-methionine and *N*-acetyl-D-phenylalanine. We were pleased to

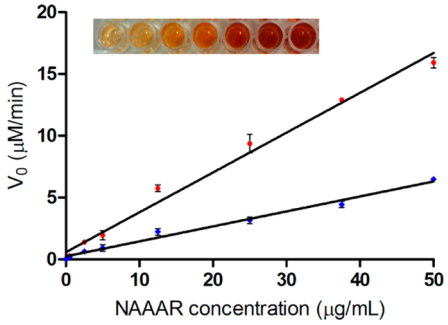

Figure 1. Activity at different concentrations of the NAAAR double mutant (G291D/F323Y) toward *N*-acetyl-D-methionine (red circles) and *N*-acetyl-D-phenylalanine (blue diamonds).

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note the amount of NAAAR required to generate an observable rate with *N*-acetyl-D-methionine, the substrate used to generate the double mutant, was 10−20 μg/mL. Moreover, we also observed a linear rate with the novel substrate *N*-acetyl-D-phenylalanine suggesting that assay would have broad substrate applicability.

These experiments are evidence that the rates are independent of the concentrations of L-acylase, L-AAO, or HRP. To further demonstrate that the components of the coupled assay do not limit the observed rate, the concentration of the coupled enzymes (L-acylase, L-AAO, and HRP) was decreased, and the reactions were repeated (Supporting Information Figure S-2). Decreasing L-acylase levels 5-fold, L-AAO levels 4-fold, or HRP levels 10-fold did not significantly affect the observed rates, thus indicating that the standard reaction conditions were sufficient and that the coupled enzymes are not limiting the observed initial rates.

Determination of NAAAR Kinetic Parameters. In order to evaluate the quality of the data resulting from the spectrophotometric assay, we determined the kinetic profile of the NAAAR G291D/F323Y mutant and compared it with those calculated by the established chiral HPLC method using *N*-acetyl-D-methionine as a substrate. A K_M value of 11.47 ± 1.36 mM and a k_{cat} value of 48.58 ± 1.64 min^{-1} were determined using the spectrophotometric assay, and the HPLC method gave values of the same order (K_M 23.41 ± 2.12 mM, k_{cat} 153.4 ± 5.4 min^{-1}, Supporting Information Figure S-3). The higher K_M obtained with the HPLC assay is due to the inherent error in measuring the formation of small amounts of product at low substrates concentrations with the HPLC analysis software. In fact, this highlights another limitation of the HPLC method; the lowest substrate concentration at which turnover can be confidently measured is only 5 mM *N*-acetyl amino acid substrate. In contrast, the spectrophotometric assay has a lower limit of detection ∼0.25 mM.

We also note that the measured k_{cat} values are lower compared to those calculated previously.[14] This is due to the modification of certain parameters in the protocol. We have replaced the previously used buffer TRIS-HCl with phosphate buffer to avoid amine contamination. It has been shown that NAAAR shows greater activity with Co^{2+} than Mg^{2+} as the divalent cation,[9] but here we used $MgCl_2$ instead of $CoCl_2$ due to solubility issues when using phosphate buffer. In this study the reactions were carried out at 50 °C, which is the maximum temperature achievable with the plate reader, instead of 60 °C. We used 49.6 U/mL L-acylase in the spectrophotometric assay, in contrast to 198.5 U/mL used in previous HPLC assays, as this was the most appropriate concentration to use based on the coupling enzyme optimization experiments (see Supporting Information Figure S-2). All of these combined factors resulted in a lower specific activity of the enzyme compared to the previous assays.

Applicability of the Coupled Assay to High-Throughput Screening of NAAAR Substrates and NAAAR Variants. With the optimized HTP method in hand, we first used it to screen the substrate profile of NAAAR and calculated the kinetic values for those that gave detectable activity (Figure 2A, Table 1). We found that *N*-acetyl-D-methionine was the best substrate for NAAAR showing k_{cat} values 3- and 12-fold higher than those obtained for *N*-acetyl-D-phenylalanine and *N*-acetyl-D-phenylglycine. However, NAAAR shows higher affinity for *N*-acetyl-D-phenylalanine than *N*-acetyl-D-methionine (K_M values of 4.20 ± 0.39 and 11.47 ± 1.36 respectively). Moreover,

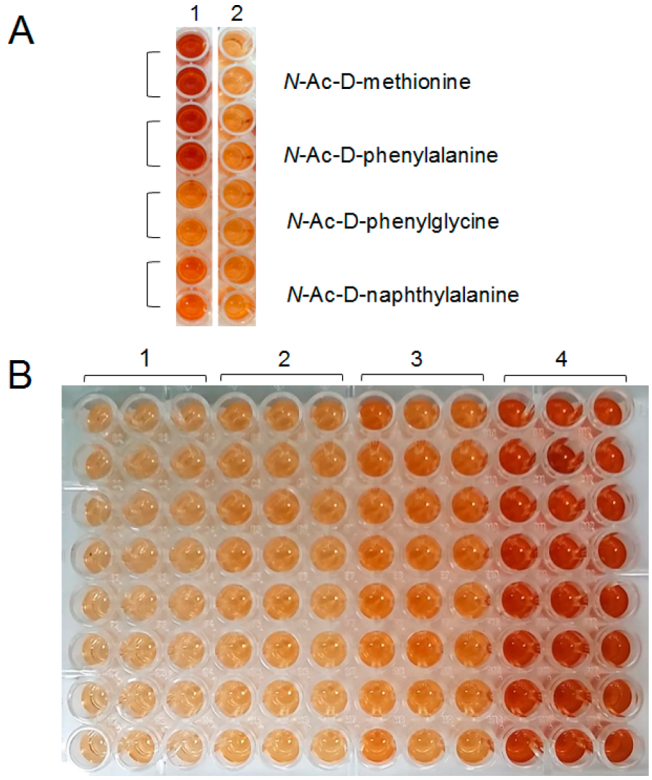

Figure 2. Control reaction plates for the NAAAR coupled assay. (A) Screening with different NAAAR substrates. Each well uses a cell-free extract from *E. coli* cells harboring the NAAAR (G291D/F323Y) expressing plasmid (column 1) or empty pET26b plasmid (column 2). (B) Comparison of the activities of *E. coli* cell-free extracts with 10 mM *N*-acetyl-phenylalanine as substrate from cells harboring (1) empty pET26b plasmid, (2) wild-type NAAAR, (3) single mutant NAAAR (G291D), and (4) double mutant NAAAR (G291D/F323Y).

the HTP assay was used to identify a novel substrate of NAAAR, the unnatural acetyl-amino acid *N*-acetyl-D-naphthylalanine, which had not been described before (Figure 2A, Table 1). NAAAR displays modest activity for this large hydrophobic amino acid (K_M 0.76 ± 0.06 mM, k_{cat} 3.55 ± 0.06 min^{-1}); however, it displays substrate inhibition above 20 mM concentrations.

The optimized spectrophotometric assay was evaluated for its suitability for HTP screening of NAAAR activity in whole cell lysates/cell free extracts. HTP screening assays are generally carried out in 96- or 384-well plate formats. Requisites for a good screening assay are robustness, reproducibility, accuracy, and adequate sensitivity in order to differentiate improved activities among a large number of samples. For proof-of-principle, several replicas of *E. coli* cells overexpressing wild type NAAAR, the single mutant NAAAR G291D, the double mutant NAAAR G291D/F323Y, and empty pET26b plasmid as a negative control, were used to select the best culture and lysis conditions (Figure 2B). Two different culture media were assayed for cell growth, Terrific Broth (TB) and autoinduction media. Autoinduction media resulted in higher protein production and avoided the addition of IPTG to induce NAAAR expression and was therefore selected for further assay optimization. Cell lysis was tested using 1 mg/mL lysozyme or BugBuster (primary amine-free) extraction reagent and benzonase as resuspension buffer. Significantly better cell lysis was achieved using BugBuster solution. Free extract were tested

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Table 1. Kinetic Parameters of *Amycolatopsis* NAAAR (G291D/F323Y)

	N-acetylated amino acid			
	D-methionine	D-phenylalanine	D-phenylglycine	D-naphthylalanine
K_M (mM)	11.47 ± 1.36	4.20 ± 0.39	8.39 ± 0.80	0.76 ± 0.06
k_{cat} (min^{-1})	48.58 ± 1.64	6.23 ± 0.24	4.00 ± 0.21	3.55 ± 0.06
k_{cat}/K_M	4.24	1.48	0.48	4.67

to select the best enzyme concentration to monitor the activity in the presence of different substrates. For this purpose, the optimized spectrophotometric assay conditions were adapted to 96-well plate format, and the assay was performed as described in Materials and Methods. The best dilution for *N*-acetyl-D-methionine was 10-fold, whereas for *N*-acetyl-D-phenylalanine a smaller, 2-fold dilution was selected.

For evaluation and validation of the HTP assay, we determined the coefficient of variance (CV) and the "*Z*′-factor" using the standard assay conditions as described in Materials and Methods. The "screening window coefficient" *Z*′-factor provides a useful tool for comparison and evaluation of the quality of assays since it reflects both the assay signal dynamic range and the data variation associated with the signal measurement.[28] *Z*′-values between 0.5 and 1 are considered a good to excellent assay, whereas *Z*′-values between 0 and 0.5 relate to double or "yes/no" type assays. To calculate the *Z*′-value, a set of control wells was prepared with cells expressing the wild type NAAAR. These give the minimum activity values and are defined as the "negative control". Similarly, wells containing the double mutant NAAAR (which we know has increased activity) are defined as "positive controls".[28] Table 2

Table 2. Evaluation of the Spectrophotometric Screening Method

	N-acetyl-D-methionine		*N*-acetyl-D-phenylalanine	
	WT	G291D/F323Y	WT	G291D/F323Y
μmol/min/mL	11.93	27.39	1.58	20.29
σ	1.11	1.22	0.15	1.89
CV (%)	9.31	4.47	10.08	9.32
Z′-factor	0.5		0.7	

shows that CV values below 10% are obtained for all sample sets (for both substrates and wild type and double mutant NAAAR), which suggests good reproducibility of the assay. Furthermore, *Z*′-values ≥0.5 are obtained for the two substrates tested confirming the suitability of the assay for further applications such as saturation mutagenesis library screening to generate further improved NAAARs.

The data obtained (Figure 2B, Table 2) show substantial differences between the activities observed for wild-type and double mutant NAAAR and confirms that this assay could have been used to detect the double mutant NAAAR (G291D/F323Y) as a positive hit from our previous mutagenic NAAAR libraries.[14] In fact, the new HTP assay was sensitive enough to pick out even the single mutant NAAAR variant (G291D) which displays only a slight increase in activity compared to the wild type enzyme (Figure 2B). The versatility of the method relies on the possibility of either detecting obvious color changes by eye, which saves a lot of time when large number of variants need to be screened (Figure 2B), but, on the other hand, it can be continuously monitored with a plate reader, which avoids many of the artifacts associated with end-point assays.

We note that Isobe and colleagues recently developed an enzyme coupled system to assay L-amino acylases.[32] In their study they purified an L-AAO from *Rhodococcus* sp. AIU LAB-3 and used the *Aspergillus* L-amino acylase (Amano) to optimize their method. They isolated >10.0 mg of L-AAO from 6 L of bacterial culture in four chromatographic steps. They chose to couple hydrogen peroxide formation to react with 4-amino-antipyrine and detected the product at 555 nm. The L-AAO displayed broad substrate specificity was most active at ∼50 °C and stable from pH 6.0−8.0 with an optimum of pH 8.0. Our study is the first to couple the NAAAR through to a chromogenic product in a HTP plate-reader format. Isobe and colleagues work suggests that it should be possible to either combine or mix and match elements from both our and their method to cover a broader range of NAAAR substrates and conditions.

CONCLUSION

In this study, we developed a continuous spectrophotometric assay for the screening of gene libraries to discover improved NAAAR variants. Moreover, the assay is also applicable for the identification of new NAAAR substrates. This assay couples NAAAR and an L-aminoacylase to yield free amino acids from their *N*-acetylated substrates. The production of the free amino acids is easily coupled with L-AAO and HRP, producing a colored product which can be spectrophotometrically monitored at 436 nm. The development of this assay has allowed us to overcome the limitations of the previous *in vivo* auxotrophic selection and HPLC-based assays.[3] We are now able to evolve NAAAR (by both rational and random methods) toward a large number of natural and non-natural amino acid substrates with the proviso that they are substrates for both coupling enzymes (*T. litoralis* L-acylase and *C. atrox* L-AAO). Limitations in the coupling enzymes used here can be overcome by selecting alternatives that best meet the requirement of the substrate of interest (e.g., the L-AAO from Isobe et al., 2013). We appreciate that the assay can only be used with D-enantiomers. To address this we are currently developing an equivalent HTP coupled assay that uses *N*-acylated L-amino acid substrates.

The optimization of the assay with purified enzymes, as well as cell-free extracts, indicates that the HTP assay is rapid, robust, versatile, and accurate. The kinetic constants calculated with the new HTP assay were comparable to those obtained by a previous HPLC method. More importantly, this assay is compatible with reagents commonly used to prepare cell-free extracts and is thus amenable to HTP screening of a large number of samples in a 96-well plate format. The assay allows rapid determination of activity by direct visualization and also possesses the advantage of continuously measuring product formation over time, minimizing the number of false positive samples. The applicability of the new HTP assay has proved to be a very useful tool in the identification of further improved novel NAAAR variants with activities toward new amino acid substrates with high commercial value.

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■ ASSOCIATED CONTENT

Supporting Information

Figure S-1. Comparison of NAAAR activity and background activity Figure S-2. Saturating conditions of coupled enzymes. Figure S-3. Comparison of K_M for *N*-acetyl-D-methionine calculated with the chiral HPLC method (A) and HTP spectrophotometric assay method (B). This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare the following competing financial interest(s): Due to the nature of the research two of the four authors are employed by Dr. Reddy's Laboratories Ltd. and have commercial interests in the use of the enzymes in this report.

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