

Rapid screening and scale-up of transaminase catalysed reactions†‡

Matthew D. Truppo,^{*a} J. David Rozzell,^b Jeffrey C. Moore^c and Nicholas J. Turner^{*a}

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A rapid, high-throughput screening methodology has been developed for the determination of transaminase activity. This pH based, colorimetric assay can also be used to scale reactions directly from 100 μ L screening scale to 25 mL development scale. Additionally, three techniques have been developed to drive transamination reactions toward complete conversion. The first method uses lactate dehydrogenase to remove the inhibitory pyruvate keto acid by-product from the reaction and drive reaction equilibrium toward the desired amine. The second method is a single enzyme system, and uses a large excess of isopropylamine to drive the transamination. Method three requires only a catalytic amount of amine donor, as an amino acid dehydrogenase is employed to regenerate the amine donor *in situ* using ammonia. All three systems have been demonstrated for the production of optically pure methylbenzylamine from acetophenone. An enantiomeric excess of >99% was achieved for both the *R*- and *S*-methylbenzylamine products.

Introduction

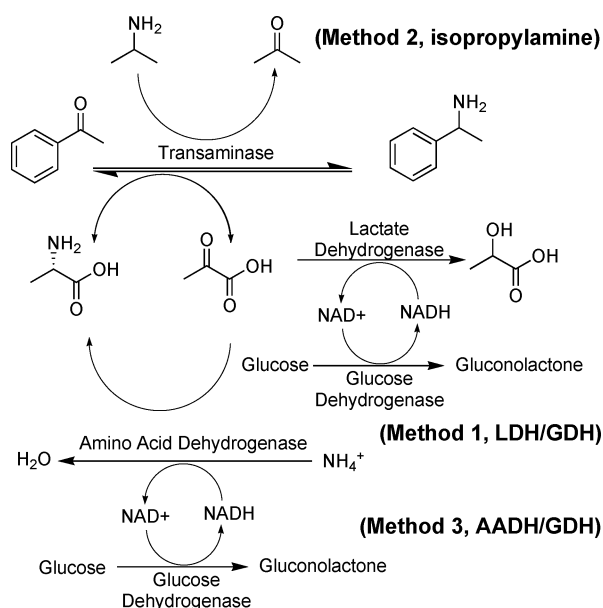
Enantiomerically pure chiral amines are valuable building blocks for the preparation of pharmaceutical agents that span a range of therapeutic areas including antihypertensives, antibiotics, antidepressants, antihistamines, and antidiabetics.¹ Although a number of enzymatic methods have been developed for the production of chiral amines with high enantiomeric excess, most processes are based upon kinetic resolution *via* enantioselective acylation using a lipase or acylase.^{2–6} Attempts to develop dynamic kinetic resolutions (DKRs) using lipases and metal catalysts have met with partial success, although the reaction conditions required for racemization of the amine are quite harsh and hence incompatible with the requirements for the acylating enzyme.^{7–9} A conceptually different approach for deracemization of amines has been developed using an amine oxidase in combination with chemical reducing agents.¹⁰

Transaminases represent an attractive option for chiral amine synthesis in that they catalyse the transfer of ammonia from an amine donor to a prochiral ketone.¹¹ Moreover, these enzymes typically possess high turnover rate, stability and a tightly bound pyridoxal cofactor. However, few examples of large-scale reactions have been reported^{12,13} largely as a result of three drawbacks that hinder their application on a preparative scale; (i) the equilibrium constant for the reaction generally favours the ketone starting material,¹⁴ (ii) screening for substrate acceptance largely

relies upon low-throughput methods such as HPLC and (iii) transaminases typically suffer significant product inhibition by the ketone or keto acid by-product.¹⁴ Although previous researchers have separately addressed the issues of screening¹⁵ and product inhibition,¹⁶ no simple procedure exists for carrying out both processes under the same conditions to allow rapid progress from small to large-scale reactions.

Results and discussion

Scheme 1 shows a transaminase catalysed reaction for conversion of acetophenone **1** to 1-phenylethylamine **2** together with three different systems that can potentially provide the amino donor. As outlined above, we envisaged developing a high-throughput



Scheme 1 Overview of transaminase catalysed reaction.

^aSchool of Chemistry, University of Manchester, Manchester Interdisciplinary Biocentre, 131 Princess Street, Manchester, UK M17DN. E-mail: Nicholas.turner@manchester.ac.uk; Fax: (+44)161 306 5173

^bCodexis Inc., 129 N. Hill Avenue, Pasadena, CA, 91106, USA

^cDepartment of Process Research, Merck Research Laboratories, Merck & Co., Inc., Rahway, NJ, 07065, USA

† Dedicated to Professor Andrew B. Holmes on the occasion of his 65th birthday.

‡ Electronic supplementary information (ESI) available: General experimental and HPLC assay conditions, rapid pH indicator based colorimetric transaminase activity assay, 25 mL scale transamination reactions, and HPLC chromatograms. See DOI: 10.1039/b817730a

screening method that operated under conditions very close to the larger scale process.

In Method 1, L-alanine is employed as the amino donor and the pyruvate which is generated is reduced by lactate dehydrogenase (LDH) to L-lactate.¹⁷ Removing the pyruvate serves the dual purpose of driving the reaction to completion and also eliminating pyruvate inhibition of the transaminase. Glucose dehydrogenase (GDH) is used to recycle the NADH cofactor as previously demonstrated for other NADH dependent processes. The LDH/GDH system leads to a reduction in the pH of the reaction through the production of gluconic acid, offering a means to (i) rapidly screen for transaminase activity by employing a pH sensitive dye and (ii) monitor the progress of the reactions at larger scale by use of automated addition of base.

Fig. 1 shows the effect of removal of pyruvate, *via* the LDH/GDH system, on the conversion of acetophenone to (*S*)-1-phenylethylamine with the (*S*)-selective transaminase ATA-103.¹⁸ In the absence of LDH, addition of one equivalent of L-alanine resulted in <0.5% conversion after 10 h. Increasing the L-alanine concentration to 10 eq. gave only a marginal improvement (3% conversion). However, addition of the LDH/GDH system to the latter reaction allowed the transamination to proceed to 70% conversion in the same amount of time.

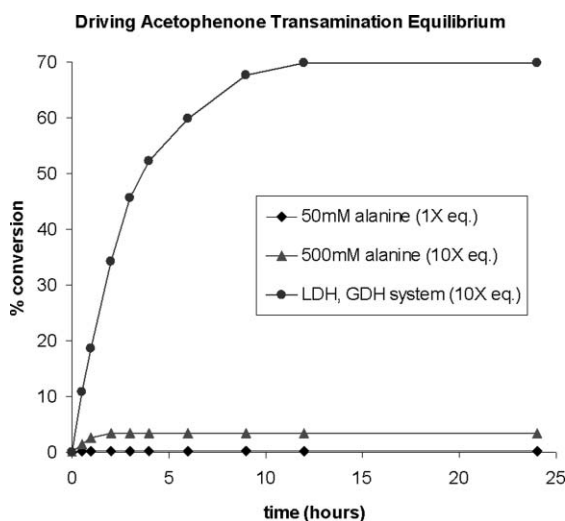


Fig. 1 Progress of 1 mL scale screening reactions using transaminase ATA-103 and 50 mM acetophenone.

To develop the screening assay, 100 μ L scale reactions were set up in a 96-well microtitre plate with a pH indicator dye (phenol red) added to the reaction mixture. The acetophenone substrate, phenol red and buffer concentrations were calibrated to allow for precise, quantitative determination of reaction rate as a function of absorbance/pH change as monitored by a plate spectrophotometer. A panel of nine different transaminases (ATAs) was then screened for activity using the pH based assay. Fig. 2 shows the comparison of the rate data obtained by the pH colorimetric screen with that obtained using conventional HPLC analysis. In all cases very good agreement (within 5%) was obtained between the pH indicator based screen and the conventional HPLC approach. Significantly, the pH screen required only 30 min for completion and consumed only 5 mg of substrate.

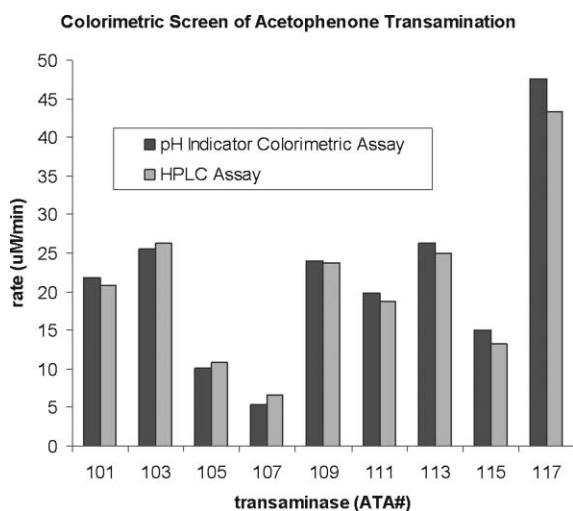


Fig. 2 Transaminase activity screen with acetophenone **1** as substrate. Comparison of rapid colorimetric pH indicator assay (100 μ L scale) reactions run in plate spectrophotometer to conventional HPLC assay (1 mL) scale reactions.

The 100 μ L scale reactions were then directly scaled to 25 mL scale and applied to the conversion of acetophenone **1** (50 mM) to both (*S*)- and (*R*)-1-phenylethylamine **2**. Progress of the reactions was monitored continuously by automated addition of NaOH (2 M) using the Mettler-Toledo Multi-Max system (Fig. 3). The NaOH addition served to keep the reaction at its optimal pH of 7.5. Under these conditions, reactions were found to proceed to >99% conversion in <10 h with both ATA-103 [(*S*)-2; >99% e.e.] and ATA-117 [(*R*)-2; >99% e.e.] (NB: for the reaction involving ATA-117, L-alanine was replaced by D-alanine as the amino donor). In both cases the product amine was isolated in 96% yield by employing a simple basification followed by extraction into *tert*-butyl methyl ether.

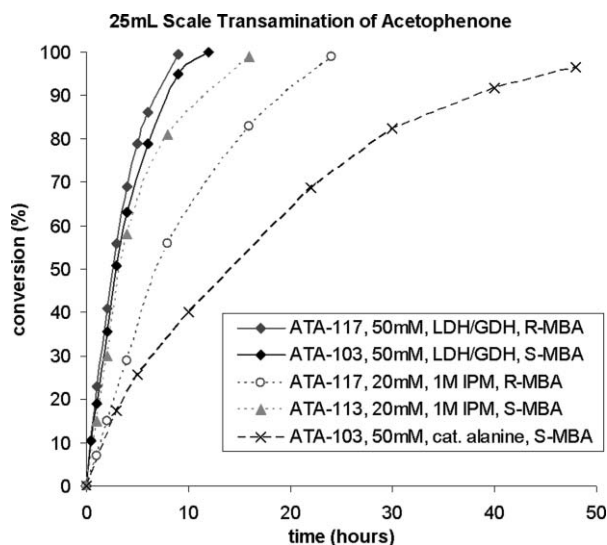


Fig. 3 25 mL scale transamination reaction progress using LDH/GDH, isopropylamine (IPM) and catalytic L-alanine reaction systems.

For comparative purposes, two additional transamination systems were investigated. Method 2 (Scheme 1) utilized a large excess

of isopropylamine (1 M) as the amino donor, instead of L-alanine, with a substrate concentration of 20 mM (Fig. 3).

Although these reactions proceeded more slowly, probably as a result of enzyme inactivation from the high concentration of isopropylamine, both (*S*)- and (*R*)-**2** were produced in 95% conversion and with >99% e.e. using transaminases ATA-113 and ATA-117 respectively. Finally in Method 3 an amino acid dehydrogenase (AADH) is added to the reaction, with NADH cofactor recycling, to convert the pyruvate back to L-alanine. Under these conditions, the ultimate amine donor now becomes ammonia and hence the process is equivalent to a reductive amination. To demonstrate the feasibility of this approach, a sub-stoichiometric amount (25 mM) of pyruvate was added to the reaction to generate the L-alanine *in situ*. The catalytic nature of the system is evident, as a reaction with 50 mM acetophenone proceeded to 96% conversion (Fig. 3).

Conclusions

In summary, we have developed a convenient and inexpensive assay for rapid screening of transaminase activity¹⁹ followed by scale-up to 25 mL scale under essentially identical conditions. This system, which should be applicable to any ketone substrate of interest, also exhibited high rates of reaction, as well as being tolerant of the high substrate charge. An alternative approach (Method 2), involving isopropylamine as the amine donor, has been demonstrated at 25 mL scale and has the dual advantage of using an inexpensive amine donor and also being a single enzyme system. Finally, an AADH system that generates catalytic L-alanine amine donor *in situ*, was demonstrated. This system, while exhibiting a slower rate than Method 1, has the advantage of using inexpensive ammonia as the effective amine donor.²⁰

Experimental

Commercial grade reagents and solvents were purchased from Sigma-Aldrich and used without further purification. All enzymes including transaminases (ATAs), glucose dehydrogenase (GDH) and lactate dehydrogenase (LDH), were generously supplied by Codexis (Redwood City, CA).

Reaction conversion was monitored using reverse phase high performance liquid chromatography (HPLC) at 210 nm using an Agilent 1100 series HPLC and a Zorbax Eclipse XDB-C18 (50 × 4.6 mm) column with a flow rate of 1 mL/min (60% acetonitrile/40% water) for 3 minutes. Enantiomeric excess was determined by normal phase high performance liquid chromatography (HPLC) at 210 nm using an Agilent 1100 series HPLC and a Chiralpak OD-H (250 × 4.6 mm) column with a flow rate of 1 mL/min (90% hexanes/10% 2-propanol) for 12 minutes. Specific rotation of the methylbenzylamine product was established by comparison to known standards.

Conventional enzyme assay

Conventional screening reactions were run at 1 mL scale in 100 mM potassium phosphate buffer using the following conditions and concentrations: 30 °C, pH 7.5, 2 g/L transaminase (ATA) enzyme, 1 g/L lactate dehydrogenase (LDH), 1 g/L glucose dehydrogenase (GDH), 9 g/L glucose (50 mM), 1 g/L NAD

cofactor, 0.5 g/L pyridoxal-5-phosphate cofactor, 45 g/L alanine (500 mM), 20 mM acetophenone. The reactions were run in 2 mL Eppendorf tubes and placed in a shaking, temperature controlled incubator (Thermomixer) at 30 °C. 40 uL samples were taken every hour to determine enzyme activity. Samples for reverse phase HPLC were diluted 1 : 10 with acetonitrile, filtered and run using the method described above. Samples for normal phase HPLC were extracted with methyl tertbutyl ether (MTBE), dried down, re-suspended in the mobile phase (90% hexanes/10% 2-propanol), and run according to the method described above.

Colorimetric transaminase activity assay

100 uL reactions were run in a 96 well microtiter plate using the following conditions and concentrations: 10 mM potassium phosphate buffer with 5% v/v MeOH, 0.036 g/L phenol red (100 μM), 1 g/L NADH, 0.5 g/L pyridoxal-5-phosphate, 9 g/L glucose (50 mM), 45 g/L alanine (500 mM), 20 mM acetophenone, 1 g/L glucose dehydrogenase (GDH), 1 g/L lactate dehydrogenase (LDH), and 2 g/L transaminase (ATA). The reactions were run at 30 °C in the plate spectrophotometer. Absorbance was measured at a wavelength of 560 nm every 30 seconds.

LDH/GDH transamination system

Transaminations of acetophenone were conducted at 25 mL scale using the LDH/GDH system under the following conditions: 100 mM potassium phosphate buffer, 1 g/L NAD, 0.5 g/L pyridoxal-5-phosphate, 18 g/L glucose (100 mM), 45 g/L alanine (500 mM), 50 mM acetophenone, 1 g/L glucose dehydrogenase (GDH), 1 g/L lactate dehydrogenase (LDH), and 5 g/L transaminase (ATA). Reactions were run at 30 °C and pH 7.5 in a Multimax[®] reactor system with overhead mechanical stirring at 400 rpm. Reaction pH was controlled through the automated addition of 2M NaOH.

Isopropylamine transamination system

Transaminations of acetophenone were run at 25 mL scale using the isopropylamine amine donor system under the following conditions: 100 mM potassium phosphate buffer, 0.5 g/L pyridoxal-5-phosphate, 1 M isopropylamine, 20 mM acetophenone, and 5 g/L transaminase (ATA). Reactions were run at 30 °C and pH 7.5 in a Multimax[®] reactor system with overhead mechanical stirring at 400 rpm.

AADH/GDH transamination system

Transaminations of acetophenone were run at 25 mL scale using the amino acid dehydrogenase/catalytic alanine system under the following conditions: 100 mM potassium phosphate buffer, 100 mM ammonium chloride, 100 mM glucose, 1 g/L NAD, 0.5 g/L pyridoxal-5-phosphate, 25 mM pyruvate, 50 mM acetophenone, 1 g/L glucose dehydrogenase (GDH), 1 g/L L-amino acid dehydrogenase (LAADH-117), and 5 g/L transaminase (ATA-103). Reactions were run at 30 °C and pH 7.5 in a Multimax[®] reactor system with overhead mechanical stirring at 400 rpm.

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