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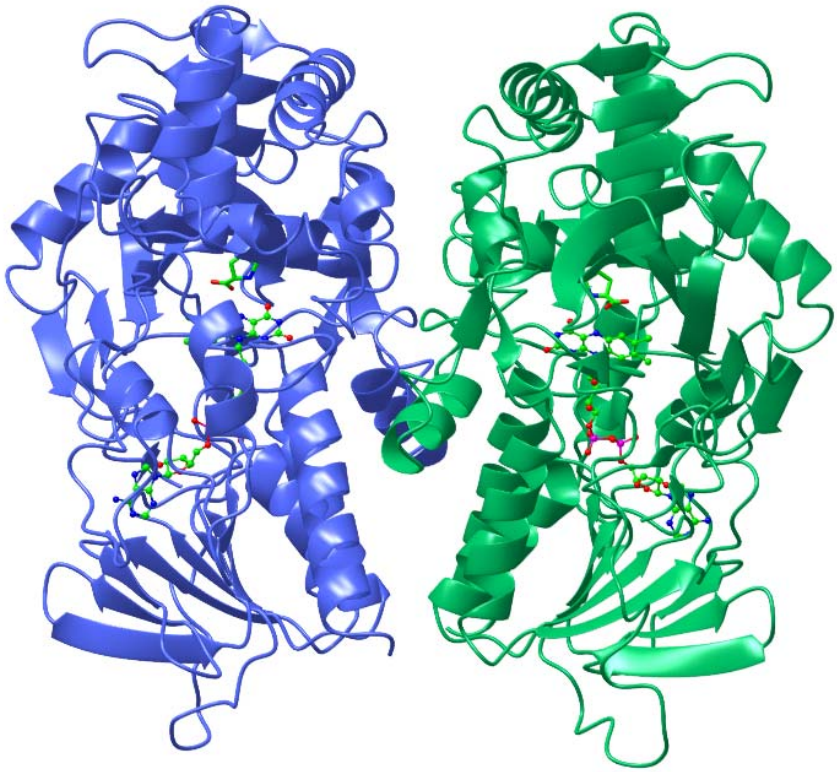

A fast and sensitive assay for measuring the activity and enantioselectivity of transaminases†‡

Jennifer Hopwood,[a] Matthew D. Truppo,[a] Nicholas J. Turner*[a] and Richard C. Lloyd[b]

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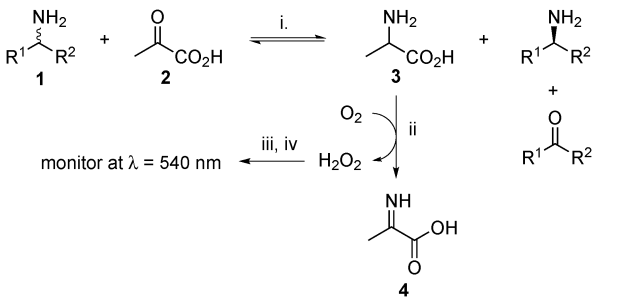
A fast and sensitive method for screening transaminase activity and enantioselectivity, using D- and L-amino acid oxidases, allows new amine substrates to be rapidly identified.

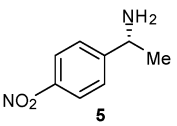
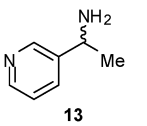
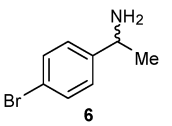
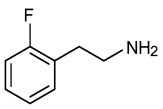
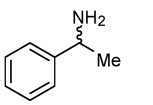
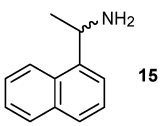
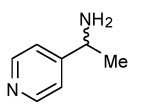
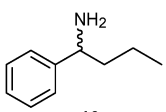
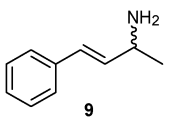
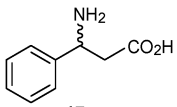
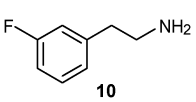
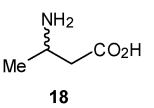
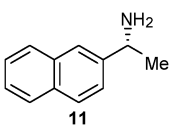
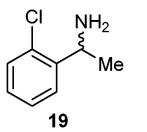
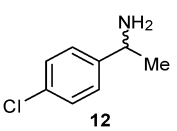
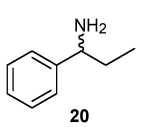
Amino transaminases (ATAs) are pyridoxal phosphate dependent enzymes that catalyse the reversible transfer of ammonia from an amine donor to a ketone acceptor. Recently they have attracted considerable interest as biocatalysts for the synthesis of enantiomerically pure chiral primary amines.[1] Particular attention has been paid to (i) overcoming problems of substrate/product inhibition, (ii) solving issues related to poor equilibrium conversions of ketone to amine and (iii) developing ATAs with broader substrate specificity.[2] Equally important is the ability to be able to rapidly screen ATAs for new substrates, with respect to both activity and enantioselectivity. Previous methods for screening for ATA activity have been based upon monitoring pH changes using the indicator dye phenyl red,[3] formation of coloured Cu–alanine complexes[4] and also an elegant UV spectrophotometric protocol.[5] Herein we describe a simple colorimetric-based method and show that it can be used to determine both the activity and enantioselectivity of a wide range of ATA substrates.

Scheme 1 outlines the basis for the new screening method. Amines **1** are treated with an ATA and sodium pyruvate **2**. Successful substrates result in conversion of sodium pyruvate to L- or D-alanine **3** which is detected by the addition of an amino acid oxidase (L- or D-AAO respectively) to the reaction mixture. Oxidation of alanine **3** to the imine **4** by AAO results in the production of H_2O_2 which can be detected colorimetrically by the addition of pyrogallol red and horse radish peroxidase.[6] Provided that all subsequent enzymes and reagents are added in excess then the ATA step becomes rate-limiting



Scheme 1 (i) Transaminase (ATA); (ii) D- or L-amino acid oxidase (AAO); (iii) horse radish peroxidase (HRP); (iv) pyrogallol red (PGR).

Table 1 Relative activity of amines with transaminase ATA-117 using the D-AAO based colorimetric assay

Amine	Relative initial rate/%	Amine	Relative initial rate/%
 **5**	139	 **13**	51
 **6**	123	 **14**	23
 **7**	100[a]	 **15**	2
 **8**	96	 **16**	1
 **9**	92	 **17**	1
 **10**	73	 **18**	0
 **11**	66	 **19**	0
 **12**	66	 **20**	0

[a] % initial rates are relative to α-methylbenzylamine.

[a] *School of Chemistry, University of Manchester, Manchester Interdisciplinary Biocentre, 131 Princess Street, Manchester M17DN, UK. E-mail: Nicholas.turner@manchester.ac.uk; Fax: +44 (0)161 306 5173*
[b] *Dr Reddy's, Chirotech Technology Ltd, Unit 162, Cambridge Science Park, Milton Road, Cambridge CB4 0GH, UK. E-mail: rlloyd@drreddys.com; Fax: +44 (0)1223 506701; Tel: +44 (0)1223 728010*

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‡ Electronic supplementary information (ESI) available: Assay conditions: sample preparation for GC and examples of spectra. See DOI: 10.1039/c0cc02919j

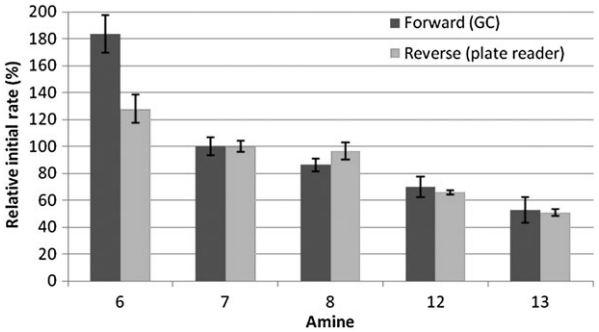


Fig. 1 Comparison of relative initial rates (relative to α-methylbenzylamine **7**) of transamination, with five different substrates, in the forward (ketone to amine: GC) and reverse (plate reader) directions.

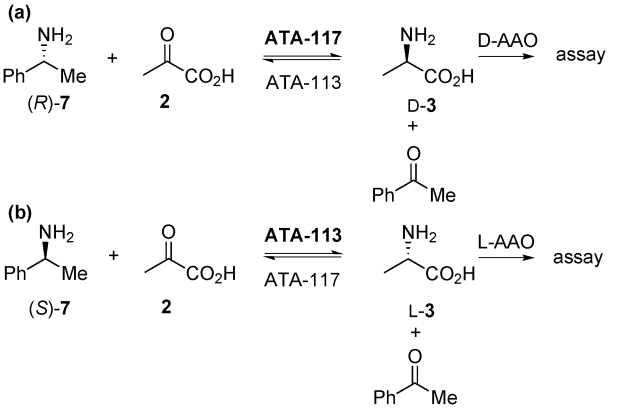
and hence its activity with respect to the amine substrate of interest can be determined.

To assess the generality of this screening method we examined a panel of 16 different amines with the (*R*)-selective ATA-117 and D-AAO (Table 1).[7,8] The entire set of substrates was screened at 5 mM concentration to obtain relative initial rates. Using this approach we were able to confidently determine initial rates as low as 1% compared to that of amine **7**.

In order to establish that the rates of the ATA catalysed reactions determined by this assay in the cleavage direction (amine to ketone) were comparable to the rates measured in the synthesis direction (ketone to amine), we selected five amine substrates **6**, **7**, **8**, **12** and **13** for study. For each substrate the initial rate (relative toα-methylbenzylamine **7**) of synthesis of the amine from the corresponding ketone was measured using a conventional GC based assay[9] and compared to the initial rates determined by the colorimetric assay. Fig. 1 shows that generally good correlation between the forward (GC) and reverse (colorimetric assay) rates of transamination was obtained confirming that the current method provides an excellent way of indirectly assessing activity in the synthesis direction. The disparity between the rates for compound **6** is currently unclear but may be due to differences in the solubility of the ketone and amine substrates.

In addition to information about relative rates of transamination it is also desirable to determine the levels of enantioselectivity of the reaction. The colorimetric assay can be easily adapted in the following way to collect such data rapidly (Scheme 2).

In the first experiment (Scheme 2a) we used (*R*)-**7** as substrate with D-AAO as the reagent to detect production of D-alanine as described above. Using transaminase ATA-117, which is known to be selective for (*R*)-configured amines, the reaction generates D-alanine and hence a signal with high intensity is observed due to the high rate of reaction (Fig. 2). If ATA-117 is replaced by the (*S*)-selective ATA-113 then the signal intensity is considerably reduced although there is indeed a measurable level of conversion. This low level of activity suggests that ATA-113 is able to catalyse transamination of (*R*)-**7**, although to a reduced amount, and correspondingly generate D-alanine. By comparison, when (*S*)-**7** is used as the substrate (Scheme 2b), with L-AAO as the enzyme for detection of L-alanine, a high initial rate is detected with ATA-113 as expected. Replacing ATA-113 with ATA-117



Scheme 2 Determination of the enantioselectivity of the transaminase catalysed reactions by using (a) D- or (b) L-AAO with the (*R*)- or (*S*)-**7** amine substrate respectively.

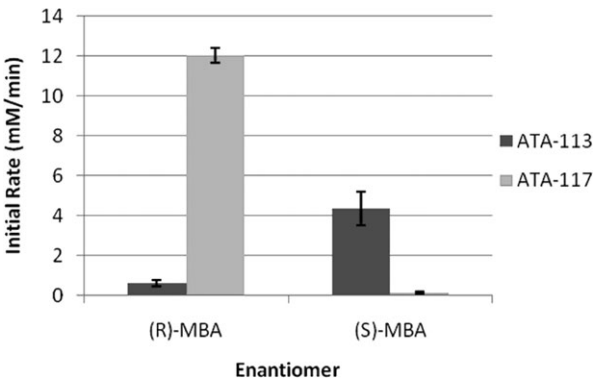


Fig. 2 Evaluation of the stereoselectivity of transamination reactions with either (*R*)- or (*S*)-**7** as substrate. For experiment with (*R*)-**7**, D-AAO was used in the assay whereas with (*S*)-**7**, L-AAO was used in the assay.

results in a very low initial rate, confirming the high stereoselectivity of ATA-117 towards (*R*)-amines. Control experiments showed that both L-AAO and D-AAO were highly enantioselective for L-alanine and D-alanine respectively. Therefore, the enantioselectivity of both ATA-113 and ATA-117 (in this example 87% ee and 99% ee respectively) can be simply estimated by comparing the initial rates of each enzyme for transamination of (*R*)- *versus* (*S*)-**7**.

In summary, we have developed a facile and rapid method for screening for transaminase activity and enantioselectivity which uses inexpensive and readily available reagents and moreover only requires a UV/Vis-plate reader to operate in 96-well microtitre plate format. The further development of this assay for high-throughput screening of transaminase libraries is currently under investigation.

Notes and references

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7 Enzymes including transaminases (ATA-113 and ATA-117), GDH and LDH were generously supplied by Codexis (Redwood City, CA). L-Amino acid oxidase from *Crotalus adamanteus* (L-AAO-A9378) and D-amino acid oxidase from porcine kidney (D-AAO-A5222) were purchased from Sigma-Aldrich.

8 *Assay conditions*: a stock solution of substrate and reagents was made up as follows; 0.1 g L^{-1} of PGR, 0.2 g L^{-1} of sodium pyruvate, and 0.05 g L^{-1} of PLP were added to 1 L of 100 mM phosphate buffer. 50 mL of this stock solution and 5 mM of amine donor (D- or L-alanine) were added to a falcon tube and the pH adjusted to 8. To 100 μL of this solution was added 10 μL of HRP (1 g L^{-1}), 20 μL of D-AAO (60 U). Finally, 100 μL of the resulting solution was dispensed into individual wells in a 96-well MTP and assays were initiated by the addition of 2.5 μL of ATA-117 (0.6 g L^{-1}) or ATA-113 (0.6 g L^{-1}). Experiments were run on a spectrophotometer at 30 °C with a wavelength of 540 nm and absorbance readings taken every 30 seconds.

9 *GC assay conditions*: an LDH/GDH system was employed to run a transamination in the forward direction. 10 g L^{-1} of glucose, 45 g L^{-1} of D-alanine, 1 g L^{-1} of NAD and 0.5 g L^{-1} of PLP were added to 16 mL of 100 mM phosphate buffer and the pH adjusted to 7.5 using sodium hydroxide (NaOH). 1 g L^{-1} of LDH, 1 g L^{-1} of GDH and 5 g L^{-1} of ATA-117 was then added to the stock solution. 0.8 mL of the stock solution was added to five different Eppendorf tubes along with 200 μL of DMSO and separately 5 mM of acetophenone, 4-chloro-acetophenone, 4-bromoacteophenone, 4-acteylpyridine and 3-acteylpyridine were added. The solutions were heated to 30 °C and agitated at 900 rpmand samples taken after 5 hours. 40 μL of 5 M NaOH was added to stop the reaction and the pH adjusted to 7 followed by extraction (3×) with 1 mL of chloroform. The remaining solution was concentrated to 1 mL and the amine acetylated with Et_3N : Ac_2O (2 : 1) and analysed *via* GC.