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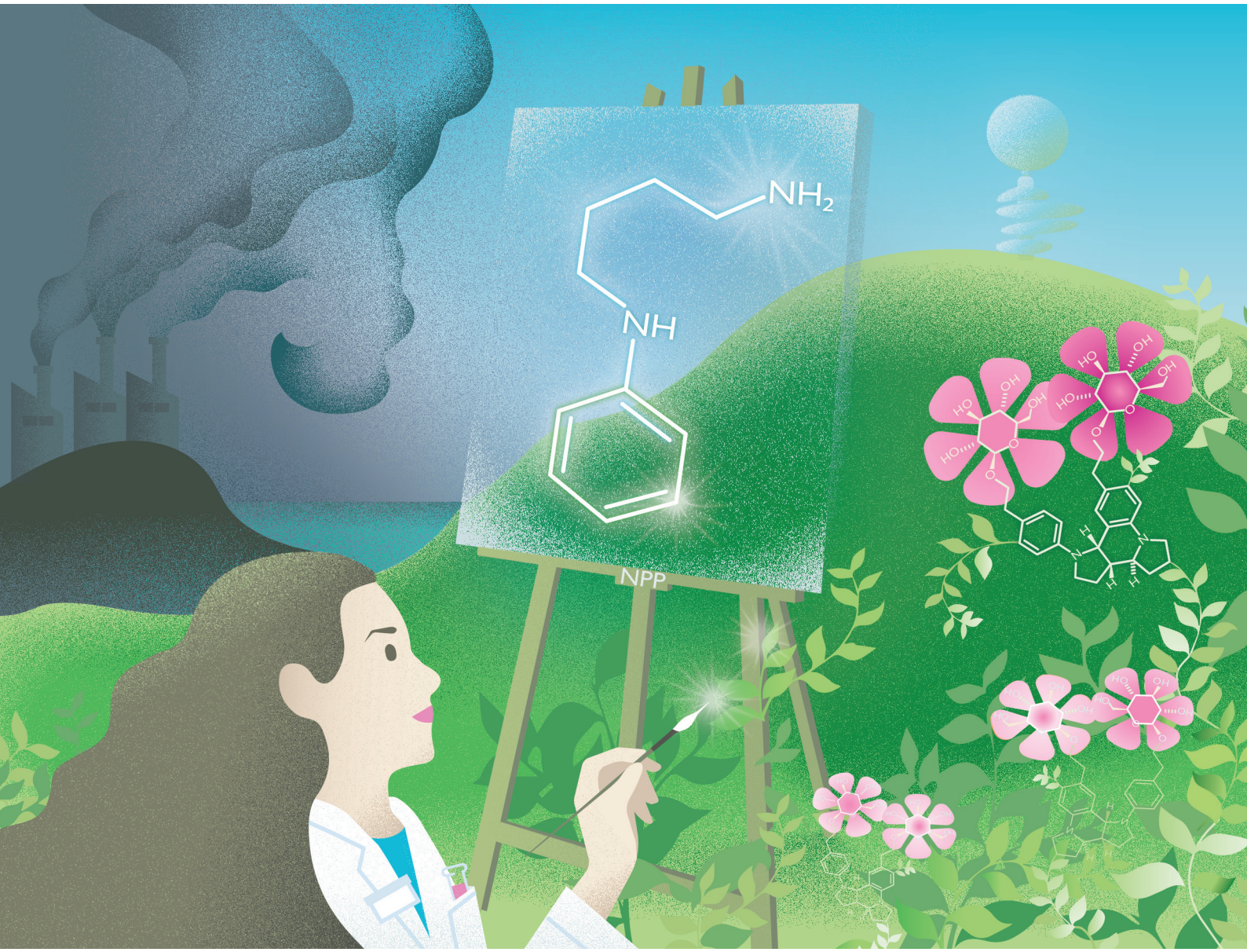

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PAPER

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N-Phenylputrescine (NPP): a natural product inspired amine donor for biocatalysis†

Catherine A. McKenna,[a] Mária Štiblariková,[b] Irene De Silvestro,[c] Dominic J. Campopiano *[a] and Andrew L. Lawrence *[a]

The synthesis of chiral amines in enantioenriched form is a keystone reaction in applied chemical synthesis. There is a strong push to develop greener and more sustainable alternatives to the metal-catalysed methods currently used in the pharmaceutical, agrochemical and fine chemical industries. A biocatalytic approach using transaminase (TA or ATA) enzymes to convert prochiral ketones to chiral amines with unparalleled levels of enantioselectivity is highly appealing. However, the use of TA enzymes in synthesis is severely hampered by the unfavourable thermodynamics associated with the amine donor/acceptor equilibrium. Several 'smart' amine donors have been developed that leverage chemical and physical driving forces to overcome this challenging equilibrium. Alongside this strategy, enzyme engineering is typically required to develop TAs compatible with these non-physiological amine donors and the unnatural reaction conditions they require. We herein disclose *N*-phenylputrescine (NPP) as a readily accessible amine donor, inspired by the biosynthesis of the dipyrroloquinoline alkaloids. NPP is compatible with a broad range of synthetically useful TA biocatalysts and performs across an unparalleled variety of reaction conditions (pH and temperature). Synthetic applicability has been demonstrated through the synthesis of the anti-diabetic drug sitagliptin, delivering the product in excellent enantiopurity using just two equivalents of NPP.

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Introduction

The need to develop more sustainable manufacturing processes in the pharmaceutical, agrochemical, and other fine-chemical industries has led to an increased interest in biocatalytic methods.[1–6] By using biocatalysts, enantiopure compounds can often be produced in high yield under very mild aqueous reaction conditions, but they can suffer from several practical limitations and drawbacks.[7,8] For example, transaminases (TAs or ATAs) provide an environmentally friendly alternative to traditional transition-metal catalysed methods for the synthesis of chiral amines, which are ubiquitous building blocks in the pharmaceutical and agrochemical industries, and find wide application as chiral auxiliaries, catalysts, ligands, and resolving agents (Fig. 1).[9,10] Unfortunately, TA-mediated transamination reactions often suffer from unfavourable reaction equilibria, an issue that can critically limit many biocatalytic methods.[11] We herein report a biomimetic solution to this problem, which will facilitate the increased use of TA biocatalysts in sustainable synthesis.

Biocatalytic transamination proceeds *via* a double-displacement mechanism (ping-pong bi mechanism), involving the

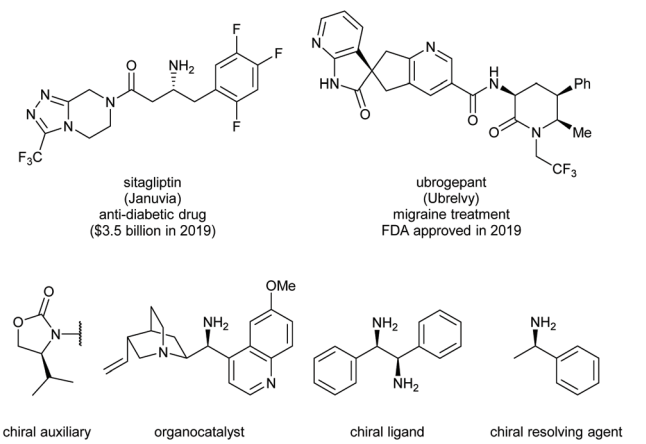

Fig. 1 Examples of chiral amine drugs, auxiliaries, catalysts, ligands and resolving agents.[12–14]

[a]*EaStCHEM School of Chemistry, University of Edinburgh, Joseph Black Building, David Brewster Road, Edinburgh, EH9 3FJ, UK.*
E-mail: dominic.campopiano@ed.ac.uk, a.lawrence@ed.ac.uk
[b]*Department of Organic Chemistry, Slovak University of Technology in Bratislava, Radlinského 9, 812 37 Bratislava, Slovakia*
[c]*Procter and Gamble, Temselaan 100, 1853 Grimbergen, Belgium*

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cofactor pyridoxal 5′-phosphate (PLP). Overall, an amino group is transferred from an amino donor to a carbonyl acceptor (Fig. 2a).[15–17] The equilibrium that exists between the donor and acceptor can be problematic when applying TA biocatalysts in synthesis, particularly when the desired reaction is enthalpically unfavourable. Previous strategies for overcoming this issue have involved the use of excess amine donor and leveraging various chemical and/or physical phenomena to drive the equilibrium forward.[18,19] The most commonly employed amine donors, despite their unfavourable reaction equilibria, are L-alanine and *iso*-propylamine (i-PrNH$_2$). L-Alanine is a widely accepted natural amine donor whose by-product, pyruvate, can be irreversibly consumed through coupled enzymatic processes. Although this is an effective strategy, it significantly increases production costs at scale.[20–23] i-PrNH$_2$ is widely used in industry because evaporative loss of the acetone by-product can be used to drive the equilibrium forward.[24,25] However, enzyme engineering is typically required to improve the stability of the transaminase in the high concentrations of i-PrNH$_2$ required to drive the equilibrium towards product formation.[26] More complex amine donors have been developed, such as *o*-2-(4-nitrophenyl)ethan-1-amine **1**, xylyenediamine **2**, and cadaverine **3**, which can be very successfully applied in specific settings (Fig. 2b).[27–34] These 'smart' amine donors each have their own niche applications, such as in colourimetric assays or when tolerance to specific reaction conditions is required, but there remains an unmet need for a widely accepted and readily available amine donor which overcomes the donor/acceptor equilibrium problem.[18,35] While i-PrNH$_2$ is currently used in industrial processes due to advancements in enzyme engineering, it is unsuitable in the asymmetric synthesis of volatile amines. In a recent review, Pavlidis and co-workers stated "…designing industrial processes with these enzymes [TAs] is still challenging, due to the fact that a universal and discernible amine donor system has not been developed".[26]

We took inspiration for the design of a new amine donor from the proposed biosynthetic pathway of the plant-derived dipyrroloquinoline alkaloids, incargranine B (**7**) and seneciopiperidine (**8**) (Fig. 2c).[36] TA-mediated oxidative deamination of an *N*-arylputrescine **4** is proposed to give an enamine **6** and an iminium ion **5** that react together through an irreversible Povarov reaction to give the dipyrroloquinoline framework. The chemical feasibility of this pathway has been demonstrated through our biomimetic total synthesis and structural revision of incargranine B **7**.[36] We herein disclose the design and synthesis of *N*-phenylputrescine **9** (NPP), which exploits this irreversible biosynthetic Povarov reaction to generate a dipyrroloquinoline by-product **10** that provides a thermodynamic driving force in the TA mediated synthesis of amines (Fig. 2d). As we envisaged, NPP **9** displaces the challenging transamination equilibrium when used in near equimolar quantities, is widely accepted by wild-type and engineered TA biocatalysts, and performs well under a broad range of reaction conditions.

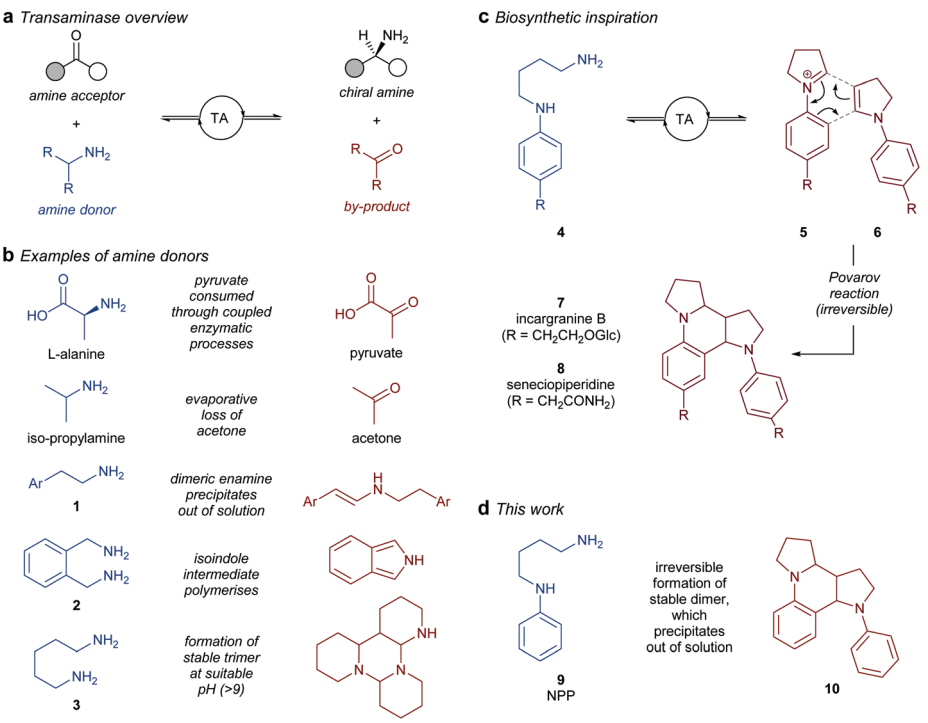


Fig. 2 (a) TA-mediated synthesis of chiral amines. (b) Example of amine donors L-alanine, i-PrNH$_2$ and **1–3** (blue) with their respective by-products (red). (c) Inspiration for this work: biosynthesis of the dipyrroloquinoline alkaloids **7** and **8**. (d) This work: a new bioinspired amine donor, NPP **9**, and the dipyrroloquinoline by-product **10**.

Results & discussion

Synthesis of NPP (9)

NPP **9** can be synthesised through a simple two-step process from commodity chemicals;[37] alkylation of aniline with 4-chlorobutyronitrile followed by reduction with borane gives NPP free base **9**, as an oil, in 80% yield on a gram scale. Alternatively, the hydrochloride salt, which is an easy to handle solid, can be similarly accessed in >80% yield in >25 g batches. This has allowed us to easily prepare over 100 g of NPP **9** salt, which can be stored under ambient conditions with no special precautions required (see ESI section 2.2 and 2.3† for details).

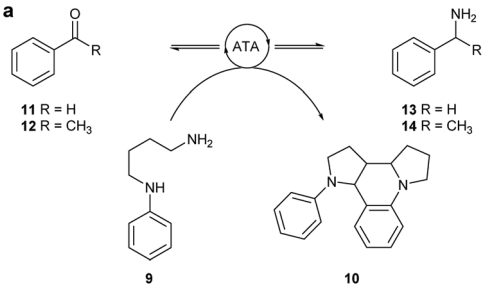
Enzyme-coupled activity screen of NPP (9)

We established NPP **9** as a suitable amine donor using *Halomonas elongata* TA (*HEWT*) with pyruvate as amine acceptor.[38,39] An adaptation of the L-amino acid oxidase (L-AAO) and horse radish peroxidase (HRP) coupled colourimetric assay was used to investigate the kinetics of L-alanine formation.[40,41] The results were fitted to Michaelis–Menten kinetics and NPP **9** displayed a K_M of 10.4 ± 1.4 mM. In comparison, (*S*)-methylbenzylamine **14**, under the same reaction conditions, has a K_M of 9.4 ± 2.2 mM (see ESI section 2.5† for more details). With evidence that NPP **9** is a good amine donor for this TA biocatalyst, our attention turned towards investigating how effective NPP **9** is at displacing the donor/acceptor equilibria with a variety of TA biocatalysts.

Quantitative screen of NPP (9) with the Codexis® ATA library

A quantitative screen (using the recommended conditions outlined in the Codexis® Amine Transaminase (ATA) screening kit) was carried out with 24 commercially available Codexis TAs and two wild type TAs (*Chromobacterium Violaceum TA* (*CVTA*) and *HEWT*) with benzaldehyde **11** as amine acceptor (Fig. 3a).[42,43] Analysis by HPLC showed all except one of the TA biocatalysts accepted NPP **9**, with 12 of the 24 Codexis TAs and both wild-type TAs giving good yields of benzylamine **13** (Fig. 3b). With this initial success, the screen was repeated with the more challenging acetophenone **12** as amine acceptor. Encouragingly, similarly effective yields of methylbenzylamine **14** product were observed (Fig. 3b). It should be noted that the reaction conditions could be optimised for each individual biocatalyst if desired. Furthermore, as new TA biocatalysts continue to be discovered through metagenomic screening and directed evolution, we envisage expanded applications of NPP **9** in the synthesis of chiral amines.[25,44,45]

During this initial screen, we noticed the formation of an off-white precipitate that correlated with activity. This observation was investigated further through a scale up of the reaction using *HEWT* with 100 mg of NPP **9**, and benzaldehyde **11** as amine acceptor. The precipitate was isolated and analysed by NMR, IR and MS (see ESI section 2.3† for more details). This spectroscopic analysis confirmed the white precipitate was the expected dipyrroloquinoline by-product **10** (dr 2 : 1), a simplified analogue of incargranine B.[36] Interestingly, the



b

R	H	CH_3
ATA-007*	0	0
ATA-013*	46	20
ATA-025	82	75
ATA-113*	90	49
ATA-117*	63	14
ATA-200*	100	18
ATA-217	33	66
ATA-234	98	93
ATA-237	96	76
ATA-238	97	91
ATA-251	96	97
ATA-254	31	49
ATA-256	24	19
ATA-260	20	38
ATA-301	9	14
ATA-303	18	31
ATA-412	28	40
ATA-415	35	42
ATA-P1-G05*	93	27
ATA-P1-F03*	96	61
ATA-P1-B04*	85	38
ATA-P2-A01*	76	21
ATA-P2-A07*	77	30
ATA-P2-B01*	41	15
HEWT	93	100
CVTA	89	96

Fig. 3 Quantitative screen of commercial and wild-type TAs with NPP **9** as amine donor. (a) TA-mediated transamination of benzaldehyde **11** (conditions: benzaldehyde **11** (10 mM) with NPP **9** (10 mM), TA (1 mg mL^{-1}), KPi (pH 8, 100 mM), PLP (0.1 mM), DMSO (10%), 30 (*) or 37 °C, 24 h) or acetophenone **12** (conditions: acetophenone **12** (10 mM) with NPP **9** (20 mM), TA (2 mg mL^{-1}) HEPES (pH 7.5, 100 mM), PLP (0.1 mM), DMSO (10%), 30 (*) or 37 °C, 48 h). (b) Tabulated HPLC yields of benzylamine **13** and methylbenzylamine **14**, relative to the best performing TAs., reactions were performed in triplicate and errors determined by standard deviation.

diastereoselectivity of this biocatalytic reaction was similar to that of our biomimetic chemical synthesis of incargranine B aglycone,[36] suggesting the TA does not provide any additional selectivity during the Povarov reaction. The fortuitous insolubility of **10** provides an additional driving force for the TA reaction and can be used as a simple visual screening method for identifying novel TA activity (see ESI Fig. S1 and S17†).

Optimising the reaction conditions of NPP (9)

The window of acceptable reaction conditions can limit the application of amine donors and hence we turned our attention towards screening the applicability of NPP **9** to prepare methylbenzylamine **14** over a range of pH and temperatures (Fig. 4a). Our previous screen identified ATA-234 and ATA-251

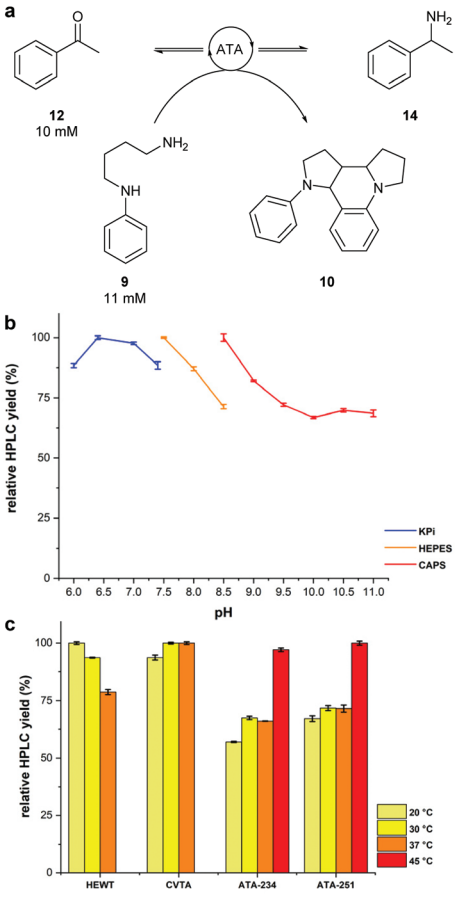


Fig. 4 NPP **9** performance over a range of pH and temperatures. (a) General scheme of the assay components were unless otherwise stated acetophenone **12** (10 mM), NPP **9** (11 mM), ATA-251 (2 mg mL^{-1}), KPi (pH 8, 100 mM), PLP (0.1 mM), DMSO (10%), 37 °C, 48 h. (b) Effects on production of methylbenzylamine **14** as pH is varied from 6–11. (c) Effects on production of methylbenzylamine **14** with NPP **9** as temperature is varied with *HEWT*, *CVTA*, ATA-234 and ATA-251 (2 mg mL^{-1}). *Methylbenzylamine **14** production by HPLC normalised relative to that of the best result., reactions were performed in triplicate and errors determined by standard deviation.

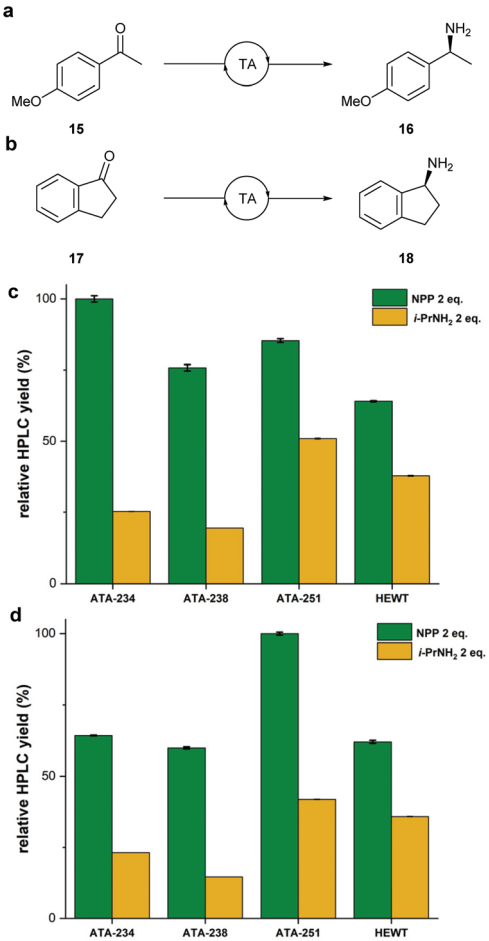


Fig. 5 Exploring NPP **9** activity as amine donor with more challenging substrates **15** and **17**. All reactions were carried out in triplicate with TA (2 mg mL^{-1}) in HEPES (pH 7.5, 100 mM), PLP (0.1 mM), DMSO (10%) at 37 °C for 48 h in a sealed system. (a) Transamination of *p*-methoxy acetophenone **15** with *i*-$PrNH_2$ or NPP **9**. (b) transamination of 1-indanone **17** with i-$PrNH_2$ or NPP **9**. (c) HPLC yield of *p*-methoxy methylbenzylamine **16** produced from transamination of *p*-methoxy acetophenone **15** (10 mM) and NPP **9** (green) or i-$PrNH_2$ (yellow) (20 mM). (d) HPLC yield of 1-indanamine **18** produced from transamination of 1-indanone **17** (10 mM) and NPP **9** (green) or i-$PrNH_2$ (yellow) (20 mM). HPLC yields of **16** and **18**, are relative to the best performing TAs. Reactions were performed in triplicate and errors determined by standard deviation.

as providing the highest yields and these commercial TAs were thus selected for further study alongside *HEWT* and *CVTA*. Pleasingly, NPP **9** performed exceptionally well across a broad range of reaction conditions when used in near equimolar quantities, relative to acetophenone **12** (Fig. 4b and c). This compares very favourably to the niche working conditions of alternative amine donors. For example, i-$PrNH_2$ needs elevated temperatures to facilitate acetone evaporation and cadaverine **3** requires high pH to drive trimer formation.[29,35,46]

Activity of NPP (9) with more challenging amine acceptors

To further explore the effectiveness of NPP **9** as an amine donor in comparison to i-$PrNH_2$, we investigated the synthesis of chiral amines from more challenging substrates such as *p*-methoxy acetophenone **15** (Fig. 5a) and 1-indanone **17** (Fig. 5b). For this screen we selected three commercial and one wild-type biocatalysts (ATA-234, ATA-238, ATA-251 and *HEWT*). We were pleased to observe NPP **9** outperformed i-$PrNH_2$ in each case under identical reaction conditions (Fig. 5c and d). This reveals the potential utility of NPP **9** in applications where i-$PrNH_2$ does not perform well. NPP **9** also outperformed cadaverine **3**, one of the most popular 'smart' amine donors used in the field, in the transamination of acetophenone **12** (see ESI section 2.17† for full details).[29,30]

Applying NPP (9) to the synthesis of an industrial target

We next selected a proof-of-concept target to highlight the practical applicability of NPP **9** in the synthesis of complex

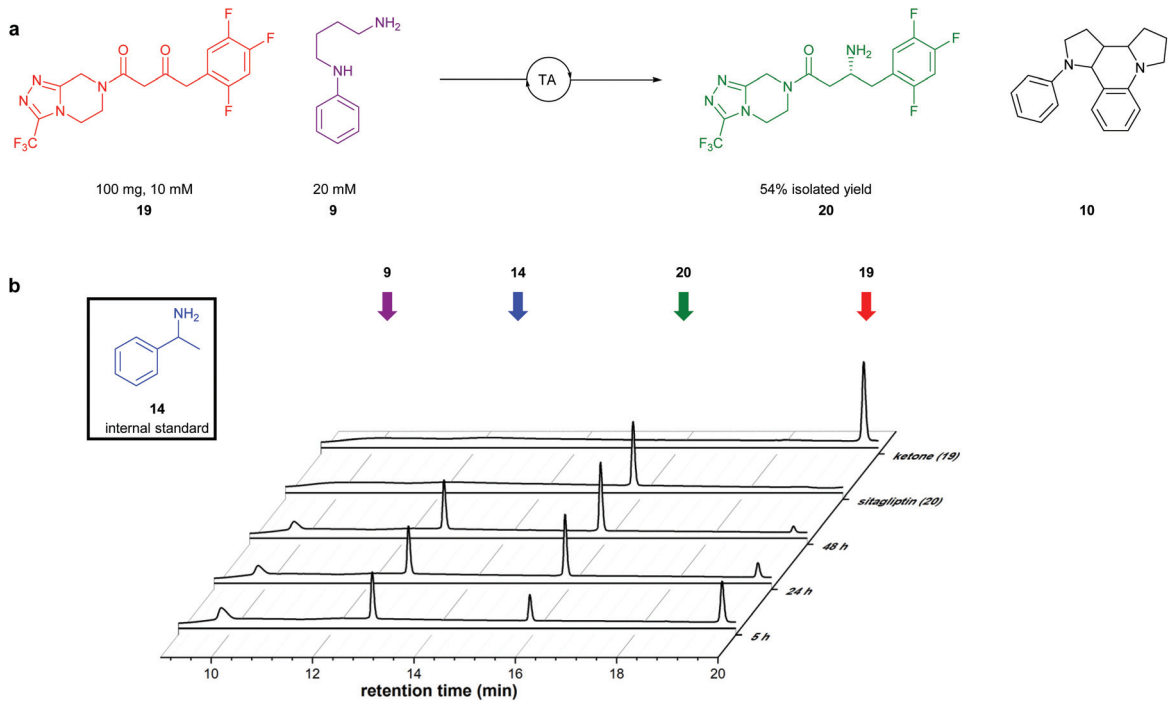


Fig. 6 Production of sitagliptin 20 with NPP **9** as amine donor. (a) Overview of the scale up reaction of ketone **19** (10 mM) with NPP **9** (20 mM), ATA-025 (2 mg mL^{-1}) in HEPES (pH 8, 100 mM), PLP (0.1 mM), DMSO (10%) at 37 °C for 48 h. (b) HPLC chromatograms of reaction samples of transamination of ketone **19** (red) with NPP **9** (purple) taken after addition of ketone at 5, 24 and 48 h in comparison to standard samples of ketone **19** and (*R*)-sitagliptin **20** (green). Methylbenzylamine **14** (blue) was used as internal standard.

targets. Merck's commercial synthesis of the antidiabetic drug, sitagliptin **20** using a late-stage TA-mediated reductive amination is perhaps the most compelling demonstration of the power of modern biocatalytic methods. This drug is manufactured from ketone **19** using a highly engineered Codexis® TA with a high molar excess of i-$PrNH_2$ as amine donor.[25] We were intrigued as to whether this process could instead be conducted using near equimolar NPP **9** as amine donor. This was achieved by initially screening the (*R*)-TAs in the Codexis® kit with ketone **19** as amine acceptor. Pleasingly five of the eleven TAs showed activity (see ESI section 2.19† for more details) with ATA-025 displaying the best conversion. An optimised reaction was carried out with ATA-025 on a 100 mg scale to give (*R*)-sitagliptin **20** in 54% isolated yield (>99% ee), with the insoluble by-product **10** easily removed by simple filtration through Celite® (Fig. 6, Fig. S42–S47 and ESI section 2.24† for more details). This showcases the significant practical advantages of using NPP **9** as a versatile amine donor in TA-mediated synthesis of chiral amines.

Conclusions

The continued demand for a universal amine donor for biocatalytic transaminations prompted us to explore how nature achieves the biosynthesis of complex natural products *via* TA-mediated pathways. Specifically, we took inspiration from the biosynthesis of the dipyrroloquinoline alkaloids **7** and **8** to design and develop NPP **9** as a new, broadly applicable amine donor. NPP **9** is easily prepared on multigram scale and displays excellent activity across an unprecedented range of pH and temperatures with both wild-type and commercially available, engineered TAs. NPP **9** works at near equimolar quantities even with challenging amine acceptors and industrially relevant substrates. The insolubility of the NPP by-product **10** allows for easy identification of TA activity and facilitates simple removal by filtration. We anticipate that the broad range of reaction conditions under which NPP **9** operates will facilitate its widespread use in the biocatalytic preparation of chiral amines. A full review of the most popular amine donors (see ESI section 2.18†) suggests that the properties of NPP **9** that we have outlined in this study will see it rapidly become the preferred amine donor for biocatalytic transamination reactions. To that end, researchers interested in using NPP **9** can contact us to request a sample.

Author contributions

The author contributions are as follows: C. A. McK. prepared NPP, carried out screening assays, analysed the ATA library and carried out preparative reactions. M. Š. optimised the synthesis of NPP and prepared the NPP salt. I. De. S. carried out the initial synthesis of NPP. D. J. C. and A. L. L. conceived and supervised the project. All authors analysed the data and contributed to the writing of the manuscript.

Conflicts of interest

The authors declare there are no conflicts of interest.

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