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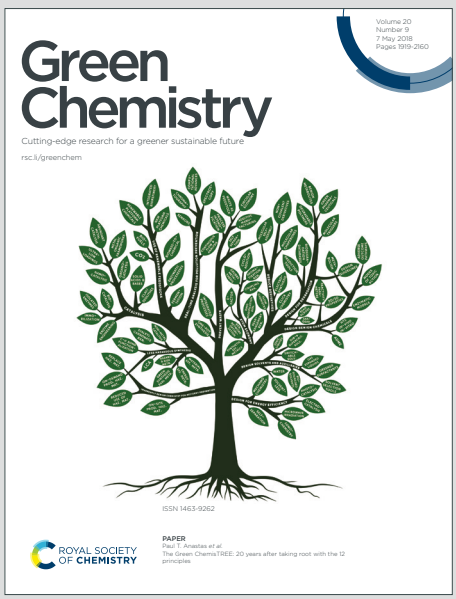

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DOI: 10.1039/C9GC02260K

COMMUNICATION

A biocatalytic cascade for the conversion of fatty acids to fatty amines

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

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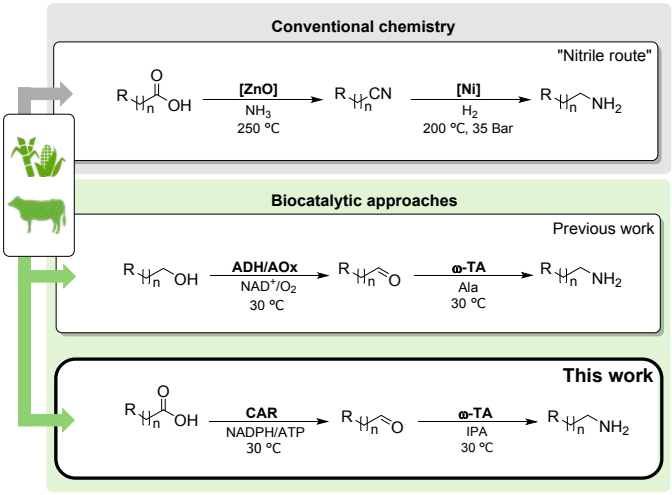
Fatty amine synthesis from renewable sources is an energetically-demanding process involving toxic metal catalysts and harsh reaction conditions as well as selectivity problems. Herein we present a mild, biocatalytic alternative to the conventional amination of fatty acids through a one-pot tandem cascade performed by a carboxylic acid reductase (CAR) and a transaminase (ω-TA). Saturated and unsaturated fatty acids, with carbon chain lengths ranging from C_6 to C_{18}, were successfully aminated obtaining conversions of up to 96%.

Amines serve as important and versatile intermediates for a wide range of chemicals synthesised by the pharmaceutical, agricultural and fine chemical industry.[1] In particular fatty amines, with carbon chain lengths ranging from C_8 to C_{22}, are utilised because of their surface active properties, which make them ideal commodity chemicals for a broad range of applications such as fabric softening, coating and corrosion inhibition, emulsification, flotation, anti-caking and crop protection.[2] The fatty amines market is estimated to be the fastest-growing amine category with an anticipated marked value of USD 2.9 billion by 2025.[3]

From an industrial point of view, fatty amines derived from renewable feedstocks are synthesised from fatty acids *via* the so-called "nitrile route".[4–8] Fatty alcohols are also used as substrates by reacting them with ammonia or alkyl amines, although this process is less commercially employed and mainly focussed on secondary and tertiary fatty amine synthesis (**Scheme 1**).[9–14] Both processes are characterised by the use of heterogeneous toxic metal catalysts (metal oxides, nickel and copper)[6,7] and harsh energetically-demanding reaction conditions (high pressure and temperature).[5] In addition, the usage of molecular hydrogen in nitrile and imine reduction steps requires additional safety measures to be implemented.[15] Another drawback of these processes is the lack of selectivity of the catalysts. Mixtures of primary, secondary and tertiary amines are commonly obtained during the reaction adding extra complexity to downstream processing.[16]

Biocatalysis is an attractive alternative for the production of bulk chemicals. The inherent mild reaction conditions together with attendant chemo- and regio-selectivity make enzymes suitable candidates for sustainable and environmentally-friendly manufacturing processes.[17] Amination of fatty alcohols has previously been achieved *in vitro* and *in vivo* by combining ω-transaminases (ω-TA) with alcohol oxidases (AOx)[18] and alcohol dehydrogenases (ADH)[19–21] respectively. Enzyme-catalysed *in situ* aldehyde generation is followed by a transamination step to yield the amine using an amino donor such as alanine. Despite the efficiency of these cascades, low enzyme activities towards non-natural substrates together with poor substrate solubility in the aqueous phase means that the synthesis of fatty amines with carbon chain lengths of C_{14} and above still remains a challenge.



Scheme 1 Chemical and enzymatic routes towards the synthesis of fatty amines from renewable vegetable oils and animal fats.

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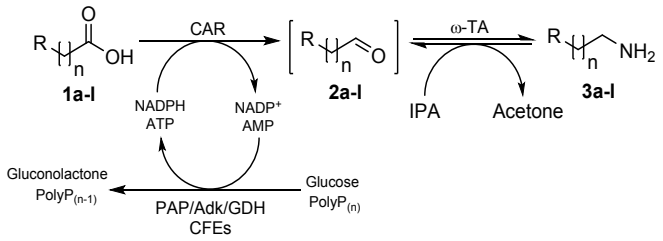
†Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/x0xx00000x

Carboxylic acid reductases (CARs) have increasingly been highlighted as attractive biocatalysts for chemical synthesis.[22–29] Through a complex 3-domain structure, CARs are capable of successfully catalysing the reduction of a broad range of carboxylic acids, yielding the corresponding aldehydes, at the expense of ATP and NADPH. The reported activity towards fatty acids[23,25,30–32] prompted us to consider CARs as suitable enzymes to access fatty amines in combination with other enzymes. The only previous report of using CARs to access fatty amines is contained in a patent wherein fatty amine synthesis is carried out through a engineered microbial metabolic pathway with carbon sources as starting materials.[33] However, the substrate scope as reported is limited to dodecylamine and a lack of quantitative data is provided.

In order to identify potentially suitable combinations of enzymes we selected four CARs (from *Nocardia iowensis,*[22] *Mycobactarium marinum,*[23] *Segniliparus rugosus*[27] and *Mycolicibacterium chlorophenolicum),* shown to be active towards fatty acids, and coupled them with six in-house ω-TAs (from *Arthrobacter sp,*[37] *Vibrio fluvialis*,[21] *Pseudomonas putida NBRC 14161,*[38] *Chromobacterium violaceum,*[18] *Agrobactarium tumefaciens*[39] *and Silicibacter pomeroyi*[40]). For each pair we examined the conversion of dodecanoic acid **1d** to doceylamine **3d** as the model reaction. Due to the poor substrate solubility under aqueous conditions, *n*-heptane containing 5 mM carboxylic acid was overlayed on the aqueous phase as a reservoir together with 5% (v/v) dimethyl sulfoxide (DMSO) to enable a gradual interphase substrate transfer. NADPH was recycled in situ by addition of glucose/ glucose dehydrogenase (GDH – Codexis CDX 901), whereas ATP was recycled by polyphosphate-AMP phosphotransferase (PAP) and adenylate kinase (Adk) from *Acinetobacter johnsonii* employing polyphosphate (PolyP) as the phosphate group donor.[41] Isopropylamine (IPA) was used in excess (50 equiv.) as amino donor for the transaminase-catalysed reaction in the presence of pyridoxal 5'-phosphate (PLP) as cofactor (**Scheme 2**).

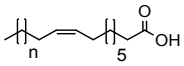
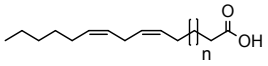
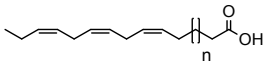


Scheme 2 Cascade design for the crossed-combination of CARs and ω-TAs with dodecanoic acid as substrate.

To our delight, most reactions resulted in the amination of **1d** to **3d** with conversions ranging from 17 to 60% (**ESI Fig. S3**). Conversion of the aldehyde to amine was dependent on the particular ω-TA, whereas CAR activities were generally comparable. The best performing pair of enzymes was shown to be ω-transaminase from *Silicibacter pomeroyi* (*Sp*-TA) and a novel CAR from *Mycolicibacterium chlorophenolicum* (*Mc*CAR), and therefore this enzyme combination was selected for further experiments. *Sp*-TA is known for having a broad substrate scope which includes aliphatic substrates.[25] *Mc*CAR has previously been identified,[25] although no biochemical characterisation has been carried out. Generating structural data could provide insight into the properties of the enzyme scaffold that make *Mc*CAR better performing biocatalyst than the rest of screened CAR enzymes.

After initial screening, subsequent reaction intensification was carried out by varying several reaction parameters including the concentration of PolyP, ATP, $MgCl_2$, glucose and IPA as well as recycling enzymes and substrate loading (**ESI Fig. S4-S9**). Conversion increased to >90% upon reduction of $MgCl_2$ concentration to near-stoichiometric amounts (1.1 equivalents) suggesting potential enzyme inhibition. In our previous work, CAR and NADPH/ATP recycling enzymes have been demonstrated to successfully perform carboxylic acid reduction with $MgCl_2$ concentrations up to 100 mM,[42] suggesting that ω-TA could be the enzyme which is inhibited. In an attempt to facilitate enzyme preparation and reduce costs of purification, we focussed our attention on using cell free extracts (CFE) instead of purified enzyme. To our surprise there was no overall negative impact on the percentage conversion when using CFEs.

Table 1. Biocatalytic amination of fatty acids[a]

Substrate	Structure	n	Conversion to amine (%)
1a		3	94
1b		5	96
1c		7	93
1d		9	92
1e		11	44
1f		13	9
1g		15	1
1h		2	89
1i		4	58
1j		6	6
1k		5	35
1l		5	63

[a]Reaction conditions: 5 mM carboxylic acid, 31.25 mM IPA, 2 mg mL^{-1} *Mc*CAR lysate, 2 mg mL^{-1} *Sp*-TA lysate, 2 mg mL^{-1} PAP lysate, 2 mg mL^{-1} Adk lysate, 0.2 mg mL^{-1} CDX-901 GDH, 0.5 mM NADP+, 0.5 mM PLP, 4 mg mL^{-1} PolyP, 25 mM D-glucose, 5.5 mM $MgCl_2$, 5% (v/v) DMSO, 100 mM pH 8 Tris buffer, 500 µL reaction volume, 500 µL n-heptane. 30 °C, 180 rpm, 20 h.

Some unwanted further reduction of the aldehyde intermediate **2** was observed (≤6%) in some of the reactions yielding the alcohol as a side by-product. This reaction is catalysed by endogenous alcohol dehydrogenases from *E. coli* present in the cell lysate. Substrates with fully-saturated carbon chains ranging from C_6 to C_{12} (**1a**-**d**) were successfully converted to the corresponding amine in high conversions (>90%). However, a reduction in conversion was observed for

substrates with saturated chains ranging from C_{14} to C_{18}, with a 46% and 8% conversion of myristic acid **1e** and palmitic acid **1f** to the amine products respectively. A negligible amount of product was obtained with stearic acid **1g**. In the case of substrates with unsaturated side-chains chains, namely myristoleic **1h**, palmitoleic **1i** and oleic acid **1j**, increased conversions were observed compared to their saturated chain analogue substrates (89% versus 44%, 58% versus 9% and 6% versus 1% respectively). The unsaturated nature of the aliphatic chain increases the substrate solubility under aqueous conditions suggesting that increased conversions may not only be due to increased enzyme activity but also to increased solubility in the reaction phase. Increasing the degree of unsaturation (linoleic **1k** and linolenic acid **1l**) further improved solubility resulting in conversions of 35% and 63% respectively. In order to confirm the reaction dependence on solubility and enzyme activity, we performed specific activity assays for substrates **1d**, **1e** and **1h** in the aqueous phase and concentrations under the solubility limit. *Mc*CAR showed different activity for each of the substrates, thus demonstrating that both the solubility and the enzyme activity play an important role in the reaction performance (**ESI Fig. S11**).

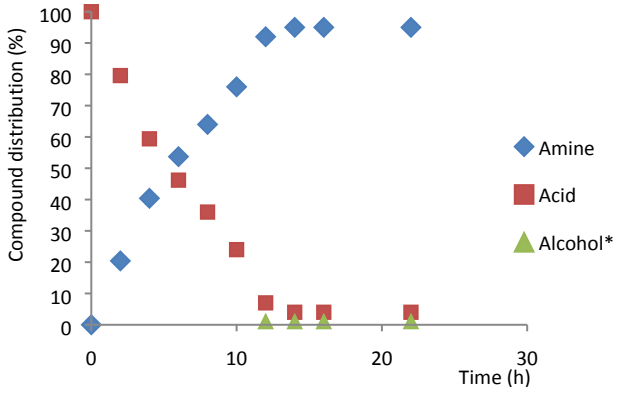


Figure 1 Reaction time-course for the amination of **1d**. *Alcohol could only be seen in late stages of the reaction, probably because its low concentration could not be detected by GC-FID.

The amination product of **1d** is dodecylamine **3d**, also known as laurylamine and commercially traded under the name of Armeen® 12D and *Adogen® 163D* among others.[43,44] In light of the high conversion obtained for this product, we further investigated the reaction by performing a 22 h time-course. Reaction completion was observed between 12 and 16 h with 95% conversion to dodecylamine (**Figure 1**). To evaluate the potential scalability and industrial applicability, a 50 mL preparative scale reaction was conducted obtaining an isolated yield of 61% after 20h. Conventional chemical methods for the scale up mass production of fatty amines can often afford high substrate loadings and equal substrate conversions regardless of the carbon chain length.[4] Different techniques can be used in order to improve biocatalytic proof-of-concept approaches and make them economically competitive alternatives to non-green chemistry routes; enzyme engineering and screening of the CAR and ω-TA sequence space would be useful approaches to find more suitable biocatalysts which can allow higher substrate loadings and a broader substrate scope. Evaluation and optimisation of the co-solvents used in the reaction could also play an important role in assuring the maximum solubility of the substrate and an optimal substrate interphase transfer.

In summary, we have designed a one-pot multi-enzyme cascade process for the amination of saturated and unsaturated fatty acids with a carbon chain length ranging from C_6 to C_{18}. Efficient cofactor recycling enzymes have been successfully coupled with *Mc*CAR and *Sp*-TA in a biphasic system with *n*-heptane as a solvent overlay and conversions up to 96% were achieved. Carbon chain length and chain unsaturation played a crucial role in substrate solubility, which is directly related to the reaction performance. To the best of our knowledge, this proof-of-concept approach represents the first *in vitro* biocatalytic cascade to access fatty amines from renewable fatty acids and the first biocatalytic cascade to access fatty amines with a chain length greater than 12 carbons.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgements

J.C. kindly acknowledge AkzoNobel for funding for a PhD studentship. J.L.G acknowledges the support of BBSRC-FAPESP Grant agreement no. BB/P01738X/1. N.J.T. is grateful to the European Research Council for an Advanced Grant funding (grant no. 742987). The authors are also grateful to Dr. Reynard Spiess for the HRMS data.

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