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# An Integrated Cofactor/Co-Product Recycling Cascade for the Biosynthesis of Nylon Monomers from Cycloalkylamines

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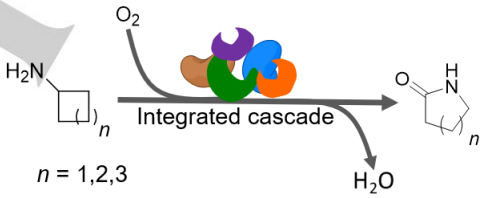
**Abstract:** We report a highly atom-efficient integrated cofactor/co-product recycling cascade employing cycloalkylamines as multifaceted starting materials for the synthesis of nylon building blocks. Reactions using *E. coli* whole cells as well as purified enzymes produced excellent conversions ranging from >80 and 95% into desired ω-amino acids, respectively with varying substrate concentrations. The applicability of this tandem biocatalytic cascade was demonstrated to produce the corresponding lactams by employing engineered biocatalysts. For instance, ε-caprolactam, a valuable polymer building block was synthesized with 75% conversion from 10 mM cyclohexylamine by employing whole-cell biocatalysts. This cascade could be an alternative for bio-based production of ω-amino acids and corresponding lactam compounds.

Biocatalysis is emerging as a valuable and efficient alternative to traditional organo- and chemo-catalytic approaches used in synthesis.[1] Multi-enzymatic, "greener", reactions have gained a surge in interest, owing to their potential for producing environmentally benign chemicals and pharmaceuticals.[1, 2] An important challenge for the development of integrated catalytic systems is access to highly specific catalysts, which can be conveniently combined in complex reaction networks, and operate under the same reaction conditions. However, the overall efficiency of these biocatalytic cascades largely depends on the availability of co-substrates and cofactors, and their proficient regeneration. Self-sufficient one-pot reactions with high atomic economy, molecular selectivity, and reduced by-product formation have been subject to a significant amount of research in recent years[2-5].

The ω-amino acids (ω-AAs) and lactams are important structural moieties present in numerous natural and synthetic bioactive compounds, and are widely used in polyamide production[1]. Nylon-6 and nylon-6,6 constitute around 90% of the total nylon produced daily worldwide; amounting to nearly 7.0 million tons annually (Grand View Research online report 2017). Industrially, nylon-6 polymer is generated via the ring-opening polymerization of ε-caprolactam, by employing cyclohexanone as a major precursor.[1a] Similarly, 6-aminohexanoic acid (6-AmHA) can be cyclized into ε-caprolactam by employing suitable ring-cyclizing enzymes, serving as valuable alternative starting material to obtain nylon 6.[6-8] Nylon 4, 5 and 6 are polyamides that can be used for the engineering of plastics, tire cords, carpeting, and food-packaging materials, owing to their superior thermal and mechanical properties.[7c-d] To date, many biocatalytic approaches for the production of ω-AAs have been developed. Kroutil and co-workers[1a] developed a multienzymatic reaction sequence, subdivided into two distinct cofactor self-sufficient cascades. This one-pot strategy resulted in 24% conversion into 6-AmHA from 50 mM cyclohexanol. Recently, some studies have been reported for the transformation of ω-AAs and ω-amino alcohols into corresponding lactams by employing various enzymes such as cyclase[9], acyl-CoA ligase[10], galactose oxidase[11], and horse liver alcohol dehydrogenase[12]. However, complete biosynthetic pathways capable of synthesizing lactams from cheap starting materials are still under development, and require well-designed reaction networks with highly active enzymes.




**Scheme 1.** General design of the integrated cofactor/co-product recycling cascade to produce nylon monomers from cycloalkylamines.

We envisaged the development of a novel biosynthetic entry for the synthesis of nylon building blocks (Scheme 1) with the following important objectives, (i) a redox self-sufficient network with high efficiency in the use of atoms, avoiding the formation of any by-product; (ii) simple and efficient transamination without using auxiliary enzymes; and (iii) shifting of the overall reaction equilibrium towards product formation. As shown in Scheme 2, we designed a smart recycling cascade employing cycloalkylamine (**1**) as a multifaceted starting material. While (**1**) served as an amine donor for the transamination, its corresponding deaminated ketone (**2**) served as a substrate for CHMO to generate lactone (**3**). Lactonase (LA) catalyzed the spontaneous hydrolytic ring-opening of **3** to obtain hydroxy fatty acid (**4**), which was further transformed to oxo-fatty acid (**5**) by aldehyde reductase (AHR). Subsequently, **5** was aminated by ω-TA to obtain ω-AA (**6**) as an intermediate and finally this open chain analogue (**6**) was cyclized into corresponding lactam (**7**) by engineered cyclase. Only stoichiometric amounts of molecular oxygen were consumed in the overall reaction process. In our previous study, ω-TA from *Silicibacter pomeroyi* (SPTA)[13] showed excellent activity towards long-chain oxo-fatty acids.

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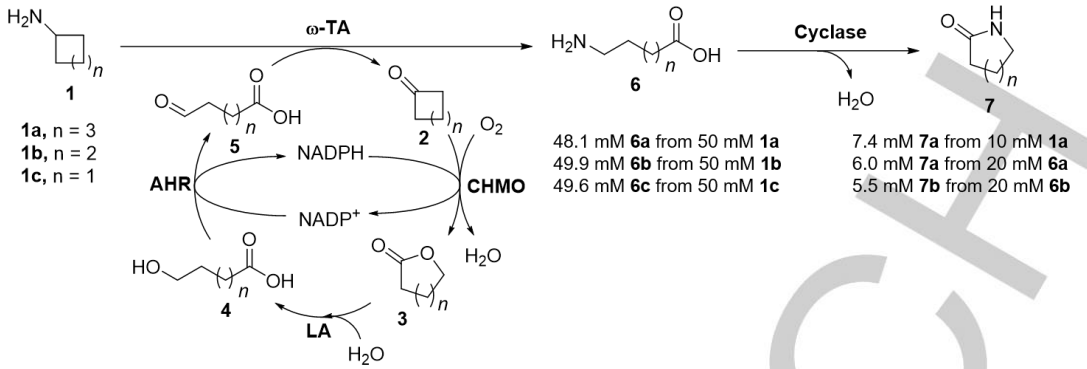


**Scheme 2.** A detailed synthetic roadmap of the smart integrated cofactor/co-product recycling cascade for biosynthesis of nylon monomers from cycloalkylamines.

We therefore examined the activity of SPTA towards three cycloalkylamines as amine donors. The enzyme showed a good preference for all substrates with specific activity of 1.93 U/mg for **1a**, 1.74 U/mg for **1b** and 1.95 U/mg for **1c**, indicating that these amines can be used as multifaceted substrates (Supporting information Figure S1). We also determined the activity of CHMO from *Thermocrispum municipale* DSM 44069 (TmCHMO)[14] towards the corresponding deaminated ketone intermediates (**2**) as substrates, and found that CHMO exhibited significant activity ranging from 0.5 – 0.6 U/mg for all intermediates (Supporting information Figure S2). We therefore initially focused on the biosynthesis of ω-AAs (**6**) and cloned all of the required enzymes, such as TmCHMO, the NADPH-dependent CHMO (double mutant C376 LM400I) from *Acinetobacter calcoaceticus* (AcCHMO),[1e,15] an LA from *Rhodococcus* sp,[16] and the NAD(P)$^+$-dependent AHR from *Synechocystis* species[8d] (Supporting information Table S1) and purified each enzyme.

In the integrated cofactor/co-product recycling cascade described here, an initiating intermediate is needed to trigger a cascade, and to generate substrates for subsequent enzymes. To identify suitable amounts of initiating intermediates, we performed reactions using varying amounts of **4a** ranging from 0.5 to 3.0 mM in the presence of 10–50 mM **1a**. The reaction performed using lower amount of **4a** showed marginally poor conversions (up to 32%; Table 1 entry 1 and 2), whereas the reaction using 3.0 mM **4a** produced 80% conversion. (Table 1 entry 3). Considering these significant results, the reaction was further examined at a higher concentration of substrate (50 mM **1a**), that led to the formation of 33.7 mM (~67%) and 25.0 mM (50%) **6a** by using TmCHMO and AcCHMO, respectively (Table 1 entry 5 and 6). Following these results, we performed further reactions using increased amount of enzymes up to 2 mg/mL of CHMO and SPTA, and 1.5 mg/mL of AHR and LA at 50 mM **1a** and 1.0 mM **4a** as an initiating intermediate. The reaction catalyzed by TmCHMO gave 42.7 mM **6a** with 85.4% conversion (Table 1, entry 7). The effect of the optimum amount of enzymes was further evaluated by using 50 mM of each of the three substrates and 3.0 mM of the initiating intermediate. Owing to the unavailability of **4b** and **4c**, we used **3b** and **3c** as initiating intermediates for **1b** and **1c** substrates. The optimized amount of enzymes boosted the conversion towards the desired products and near complete conversions (>99%) for **6b** and **6c** and 96% of **6a** were achieved using TmCHMO (Supporting information Figure S3 and Table 1 entry 8). Reactions performed by AcCHMO resulted in overall conversion of 90% in all three cases. The better conversions attained in the case of TmCHMO could be attributed to its high thermostability and high affinity towards deaminated ketones, with a $K_m$ value <0.001 mM for **2a** compared to 0.009 for AcCHMO.[10]

**Table 1.** Transformation of **1a** into **6a** at varying amounts of initiating intermediate.

| Entry | **1a** (mM) | **4a** (mM) | CHMO | Conversion. **6a** (%) | Remaining **4a** (mM)[c] |
|---|---|---|---|---|---|
| 1[a] | 10 | 0.5 | TmCHMO | 15.3±0.7 | 0.59±0.007 |
| 2[a] | 10 | 1.0 | TmCHMO | 32.0±3.0 | 0.84±0.007 |
| 3[a] | 10 | 3.0 | TmCHMO | 80.1 | 2.90 |
| 4[a] | 10 | 3.0 | AcCHMO | 70.1 | 2.86 |
| 5[a] | 50 | 3.0 | AcCHMO | 50.0 | 3.50 |
| 6[a] | 50 | 3.0 | TmCHMO | 67.0 | 2.71 |
| 7[b] | 50 | 1.0 | TmCHMO | 85.4 | 2.83 |
| 8[b] | 50 | 3.0 | TmCHMO | 96. ± 2.8 | 2.32 ± 0.01 |

[a]Reaction conditions: CHMO (1.0 mg/mL), SPTA (1.0 mg/mL), AHR (0.5 mg/mL), LA (0.5 mg/mL), 1.0 mM NADP$^+$, 0.1 mM PLP, 100 mM Tris-HCl buffer (pH 8.0) at 25°C.

[b]Reaction conditions: CHMO (2.0 mg/mL), SPTA (2.0 mg/mL), AHR (1.5 mg/mL), LA (1.5 mg/mL), 1.0 mM NADP$^+$, 0.1 mM PLP, 100 mM Tris-HCl buffer (pH 8.0) at 25°C.

[c]The other reaction intermediates were below the limit of detection.

The reactions at elevated substrate concentration (100 mM **1a**) afforded the ~56 mM of corresponding ω-amino acids. This diminished conversion can be attributed to the complex substrate and/or intermediate inhibition pattern exhibited by any of the constituent biocatalysts of the cascade. The representative upscaled reactions for the isolation and purification of the desired products (**6**) were performed using purified enzymes (5 mL total volume), and similar conversions were obtained as those for the reactions performed in a total volume of 1 mL (Supporting information Figure S3). ω-AAs products are insoluble in the organic solvents, making their isolation difficult. Therefore, a small

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chemical modification, such as the introduction of protecting groups, would alter the solubility of the desired target products (**6**). After completion of the reactions, the aqueous reaction mixtures containing the desired ω-AAs were subjected to *tert*-butyloxy carbonyl (*Boc*) protection using a well-established protocol.[17] The desired *Boc*-protected products were isolated by extraction using organic solvents, purified and characterized using GC-MS and $^1$H NMR spectroscopy, with overall isolated yields varying from 62 to 66% (See experimental section). Finally, to demonstrate the synthetic applicability of this recycling cascade on a preparative scale, the production of **6a** was carried out (196 mg of substrate (50 mM), 25 mL total volume) under optimized conditions, which led to more than 97% conversion with around 83% isolated yield.

Having established this ω-AAs producing cascade, we focused on transforming these ω-AAs into corresponding lactams, the desired target products. Recently, Lee and co-workers[10] have reported a mutant of cyclase from *Citrobacter freundii* (Cy-CF_Y152A) which could catalyze the ring-cyclization of short chain ω-AAs into corresponding lactams. We therefore screened and selected five cyclase candidates, *Citrobacter freundii* (Cy-CF_Y152A), *Enterobacter kobei* (Cy-EK), *Pluralibacter gergoviae* (Cy-PG), *Pantoea sp. Seng* (Cy-Pse) and *Pseudomonas stutzeri* (Cy-Pst) from the GenBank database (Supporting information S4 and S5). These five enzymes were tested for ring-cyclization reactions at 10 mM of **6a** as a model substrate. All of the tested enzymes displayed comparable activity for target lactam formation (**7a**) wherein, Cy-EK, Cy-Pst and Cy-CF_Y152A afforded significant conversions (>10%). Therefore, these three enzymes were selected for further research (Supporting information Figure S6).

As described by Lee and co-workers, the Y152 site was expected to be the key residue to improve the ring cyclization. We, therefore constructed Y152A variants of Cy-EK and Cy-Pst (Supporting information Figure S7). Both the variants—Cy-EK_Y152A and Cy-Pst_Y152A—improved the lactam formation up to 4.4 mM and 3.8 mM, respectively. While Cy-EK and Cy-Pst wild type enzymes produced 2.8 mM and 2.4 mM of lactam, respectively using 10 mM of **6a**. These new wild type enzymes appeared to be more active than a previously reported Cy-CF_Y152A mutant which afforded less than 1.52 mM of the desired product (**7a**) (Table 2 entry 2). Nevertheless, increasing the substrate concentration up to 20 mM (**6a**) produced 6.0 mM and 4.2 mM of desired lactam (**7a**) from Cy-EK_Y152A and Cy-Pst_Y152A (Table 2 entry 3). To check the scope of the lactamization for ω-AAs substrates, **6b** and **6c** substrates were tested, revealing that Cy-EK_Y152A had significant activity towards **6b**, with the highest yields being 5.5 mM. While **6c** was found to be least preferred substrate for all the tested enzymes (Table 2 entry 4 and 5). Next, reactions of the Cy-EK_Y152A variant were combined with the established ω-AAs producing cascade. This one-pot two-step reaction of 50 mM **1a** produced 47.0 mM (95%) **6a** intermediate, which was subsequently cyclized to produce lactam in the second step. After completion of the first step, the reaction was diluted, to achieve 10 mM of **6a** as a final substrate concentration. The desired amount of Cy-EK_Y152A and buffer was added thenceforth to adjust the final volume. This reaction successfully produced 4.42 mM of **7a** (44% conversion from initial 50 mM **1a**) indicating that Cy-EK_Y152A efficiently catalyzed ring-cyclization of ω-AAs into the corresponding lactam product (**7**) (Supporting information Figure S8). The reaction was performed by employing **1b** and **1c** as substrates, which resulted in more than 90% conversion to the corresponding intermediates (**6**), and the production of 0.68 mM and 0.34 mM corresponding **7b** and **7c** lactams (Supporting information Table S3). This mediocre conversion could be attributed to lower affinity of Cy-EK_Y152A towards **6b** and **6c** substrate or enzyme inhibition by any reaction intermediates. In fact, *in-situ* removal of the lactam product by means of biphasic reactions would help to circumvent the product inhibition but high-water solubility of the lactam and fine tuning of enzyme biocatalysts with organic solvent should be considered in the process.

Since whole-cell bio-transformations are cost-effective and advantageous regarding stability of enzymes,[3e-f,18] we tried to develop a whole-cell *E. coli* system comprising various vector combinations (Supporting information Figure S9). In order to achieve efficient recycling of the nicotinamide cofactor, the CHMO and AHR were co-expressed in the same cell. Among the different vector systems developed, a combination of pET24ma and pETduet1 for AcCHMO and AHR (AcCHMO/AHR)[19] in one cell and ω-TA and LA (ω-TA /LA) in another cell, and cyclase cloned in pET24ma was found to be suitable (Supporting information Figure S10-S13). While the expression of TmCHMO cloned in pET24ma was good, co-expression with AHR resulted in poor expression. Since the combination of TmCHMO/AHR was unsuitable, a combination of AcCHMO and AHR was used for further reactions. In order to optimize whole cell biotransformation, as described earlier, an initiating intermediate was needed to trigger a cascade. *E. coli* cells contain enough pyruvate to provide an initiating intermediate to start the process. However, the reactions performed without adding any initiating intermediate resulted in very poor conversion (0.82 mM from 20 mM **1a**).

**Table 2.** Cyclase catalyzed synthesis of lactams (**7**) from corresponding ω-AAs (**6**)[a]

| Entry | Substrate | Substrate Concentration (mM) | Cyclase (mg/mL) | Produced lactam (**7**) (mM) | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | Cy-CF_Y152A | Cy-EK WT | Cy-EK_Y152A | Cy-Pst WT | Cy-Pst_Y152A |
| 1 | **6a** | 5 | 0.2 | 0.19 | 0.21 | 0.24 | 0.18 | 0.22 |
| 2 | **6a** | 10 | 5 | 1.52 | 2.8 | 4.4 | 2.4 | 3.8 |
| 3 | **6a** | 20 | 5 | n.d | n.d | 6.0 | n.d | 4.2 |
| 4 | **6b** | 20 | 5 | 2.38 | 1.60±0.81 | 5.53±0.07 | 2.22 | 1.69±0.87 |
| 5 | **6c** | 20 | 5 | <0.17 | - | 0.41 | - | <0.11 |

[a]Reaction condition: 100 mM Tris-HCl buffer (pH 8.0) at 30 °C. n.d = not determined

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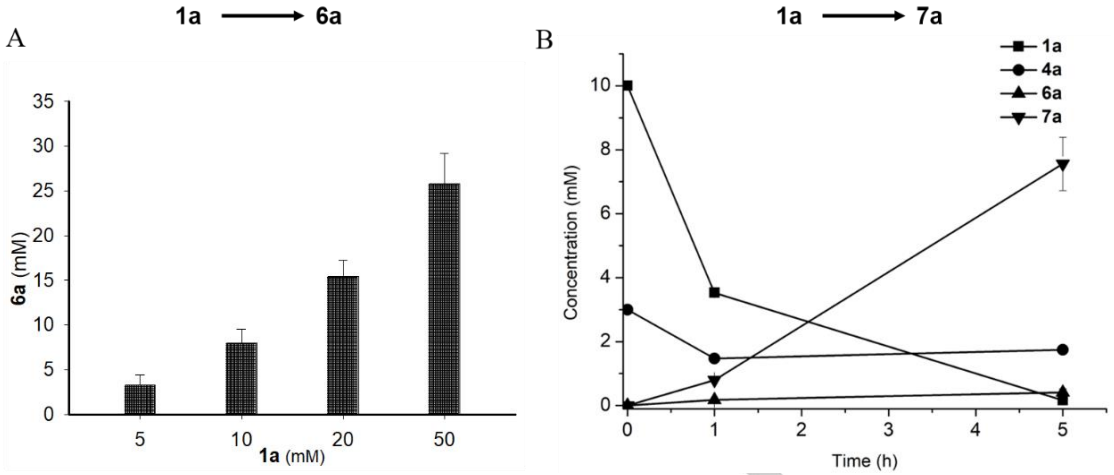


**Figure 1.** Whole cell catalyzed reaction conversions A) Synthesis of ω-AAs (**6**) from corresponding cycloalkyl amines (**1**). Reaction conditions (3 mL total volume): 10- 50 mM **1a**, 3 mM **4a**, AcCHMO/AHR (40 $mg_{DCW}$/mL), SPTA /LA (30 $mg_{DCW}$/mL), 100 mM Tris-HCl buffer (pH 8.0) at 25°C. * Approx. 2.5–2.9 mM of **4a** was detected after completion of each reaction at 24 h B) Transformation of cycloalkyl amines (**1**) into corresponding lactams (**7**). Reaction conditions (3 mL total volume): 10 mM **1a**, 3.0 mM **4a**, AcCHMO/AHR (30 $mg_{DCW}$/mL); SPTA /LA (20 $mg_{DCW}$/mL), Cy-EK_Y152A (40 $mg_{DCW}$/mL), 100 mM Tris-HCl buffer (pH 7.5) at 30°C.

Therefore, to identify a suitable initiating intermediate, three different whole-cell reactions were performed using 1.0 mM **2a**, **3a,** or **4a** as initiating intermediates in the presence of 10 mM **1a** as model substrate, and 20 $mg_{DCW}$/mL of whole-cell biocatalysts. We found that **3a** and **4a** were suitable, with conversions ranging from 2.5 to 2.6 mM respectively, whereas **2a** resulted in slightly lower product formation (~2.1 mM) (Supporting information Figure S14). Therefore, **4a** was selected as an initiating intermediate and slightly higher amounts (3 mM) were used in subsequent experiments. AHR- and LA-catalyzed reactions are well established with respect to specificity and activity towards the desired target substrates.[1,2c] Thus, transamination of **1** becomes a key step for the efficient synthesis of **6** using this recycling cascade. In search of a more efficient and selective biocatalyst for transamination, we compared the efficiency of SPTA with two other ω-TAs from *Shimia marina* (SMTA) and *Phaeobacter porticola* (PPTA). Amongst the tested ω-TAs SPTA showed formation of ~16 mM **6a.** Therefore, SPTA was selected for further reactions (Supporting information Figure S15). To evaluate the effect of varying amounts of whole-cell catalysts on the formation of desired model product **6a**, reactions were performed at different concentrations of whole cell biocatalysts. Amongst those tested, combination C attained the maximal conversion of ~16 mM (~2-fold higher than combination A, (Supporting information Figure S16). We then examined the effect of varying substrate concentration, wherein 66% (3.3 mM) of **6a** could be generated from 5 mM **1a**. The reactions performed at 10- and 20-mM concentrations yielded nearly 80% conversion, which was 1.2-fold higher than that produced by earlier reactions. Finally, the reaction performed at elevated substrate concentration (50 mM **1a**) resulted in 52% conversion to **6a** (25.8 mM). Unlike *prim*-ADH,[1] the AHR was not inhibited by the free carboxylic acid intermediate (**4**) in this cascade (Figure 1A). To examine the performance of the whole cell *E. coli* expressing desired cyclase for ring-cyclization reaction in a one-pot, one-step manner. A reaction was performed using 10 mM **1a** substrate and the results showed that Cy-EK_Y152A efficiently catalyzed the ring cyclization of **6a** intermediate to produce ~7.4 mM (74%) desired lactam **7a** as final product (Figure 1B). Similarly, the reactions performed using **1b** and **1c** as substrates afforded the conversion of 0.82 mM and 0.41 mM of **7b** and **7c**, respectively (Supporting information Table S2). In multiple enzymatic reactions, generating a driving force for the forward reaction is a key process. The reactions catalyzed by CHMO and LA in the present cascade are practically irreversible. The deaminated products are detrimental in transamination reactions, because they impede the reaction equilibrium and also inhibit the enzymes, thereby necessitating instant removal to shift the equilibrium towards product formation.[2d-h]

In summary, we have for the first time developed a novel one-pot integrated catalytic system, recycling cofactor/co-product to produce lactams by using cycloalkylamines as multifaceted substrates. Only molecular oxygen was consumed, resulting in a highly atom-efficient approach, with no requirement for an external hydride source such as glucose or formate, or additional auxiliary enzymes. The reactions employing whole cells as well as purified biocatalysts showed excellent results with overall conversions of 80% and >95% for ω-AAs (**6**) and 75% and 44% for the corresponding caprolactam (**7a**) products, respectively. This one-pot biocatalytic route paves the way for expanding a toolbox of recycling cascades to obtain versatile polymer building blocks.

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**Keywords:** Integrated multienzyme catalysis • atom efficiency • cofactor/co-product recycling • lactams • nylon monomers

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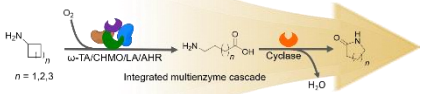


A novel, one-pot integrated catalytic system to produce the direct precursors of nylon polymer using cycloalkylamine as multifaceted and cheaper substrate is reported. The work reported herein could be a sustainable alternative to produce various ω-amino acids and corresponding lactam compounds, which generally require harsh conditions to produce by chemical means.