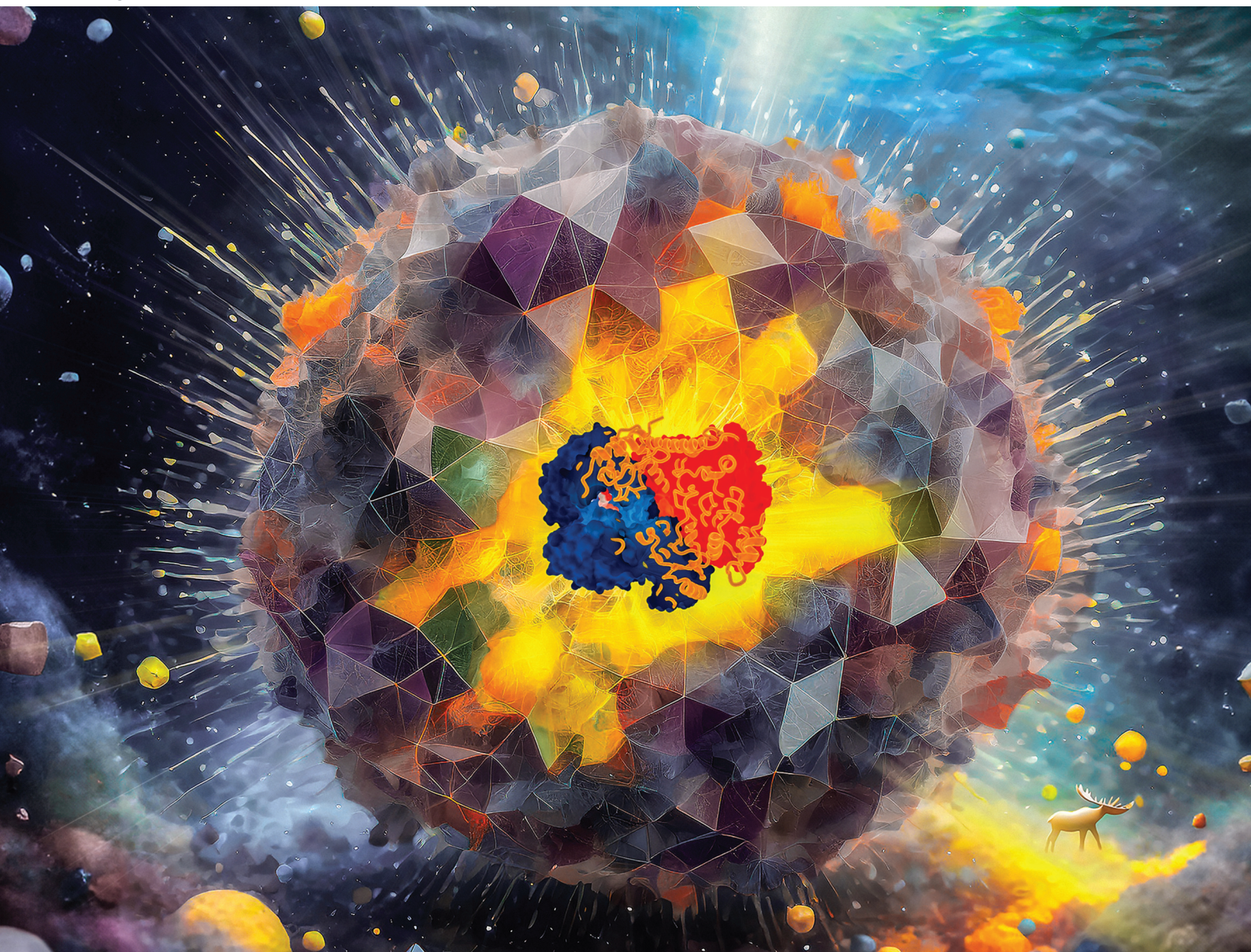


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PAPER

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Mild hydrolysis of chemically stable valerolactams
by a biocatalytic ATP-dependent system fueled by
metaphosphate



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Medium-sized 5- and 6-membered ring lactams are molecules with remarkable stability, in contrast to smaller β -lactams. As monomers, they grant access to nylon-4 and nylon-5, which are alternative polyamides to widespread caprolactam-based nylon-6. Chemical hydrolysis of monocyclic γ - and δ -lactams to the corresponding amino acids requires harsh reaction conditions and up to now, no mild (enzymatic) protocol has been reported. Herein, the biocatalytic potential of a pair of heterologously expressed bacterial ATP-dependent oxoprolinases – OplA and OplB – was exploited. Strong activity in the presence of excess of ATP was monitored on δ -valerolactam and derivatives thereof, while trace activity was detected on γ -butyrolactam. An ATP recycling system based on cheap Graham's salt (sodium metaphosphate) and a polyphosphate kinase allowed the use of catalytic amounts of ATP, leading to up to full conversion of 10 mM δ -valerolactam at 30 °C in aqueous medium. Further improvements were obtained by co-expressing OplA and OplB using the pETDuet1 vector, a strategy which enhanced the soluble expression yield and the protein stability. Finally, a range of phosphodonors was investigated in place of ATP. With acetyl phosphate and carbamoyl phosphate, turnover numbers up to 176 were reached, providing hints on a possible mechanism, which was studied by ^{31}P -NMR.

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Polyamides are synthetic polymers obtained by the repetition of units linked by amide bonds and broadly described as nylon. Based on the repeating units, different types of nylon are obtained, such as the emblematic nylon-6, predominantly used in the textile and plastic industries and produced through the ring-opening polymerization of ϵ -caprolactam.^{1,2} Nylon-5 is obtained in a similar manner from δ -valerolactam and displays ferroelectric properties and high melting point above 250 °C.³ Nylon-5,6 is obtained by the copolymerization of ϵ -caprolactam and δ -valerolactam and exhibits superior properties as fabric in terms of elastic recovery and moisture-absorbance properties.^{4,5} The manufacturing of these polymers implies problematic consequences for the environment

through the release of unreacted monomers§ into the wastewater, which thus needs to be treated before release.⁶ While biological treatments of these wastewaters are still rare, microorganisms able to degrade ϵ -caprolactam have been identified.⁶ Such approach is more challenging in the context of medium-sized lactams, especially γ -butyrolactam and δ -valerolactam, because of their exceptional chemical stability connected to the low ring strain. The increased resonance stabilization of the amide bond and the higher partial C–N double bond character in these lactams are responsible for the resistance of the carbonyl group to hydrolysis, and in general, to nucleophilic attack.^{7,8} The hydrolysis of such molecules requires in fact harsh reaction conditions, such as boiling under reflux in strongly acidic solutions for extended times.

Biotechnological access to δ -valerolactam is currently being investigated as an approach to accessing bio-sourced plastics.⁹ However, the challenging biodegradability of this molecule may hamper its wider acceptance. To the best of our knowledge, no enzymatic approach has been identified for the mild

‡ Equal contributions.

§ Hazard pictograms are reported for both γ -butyrolactam (GHS07 and GHS08) and δ -valerolactam (GHS07), see ESI† for details.

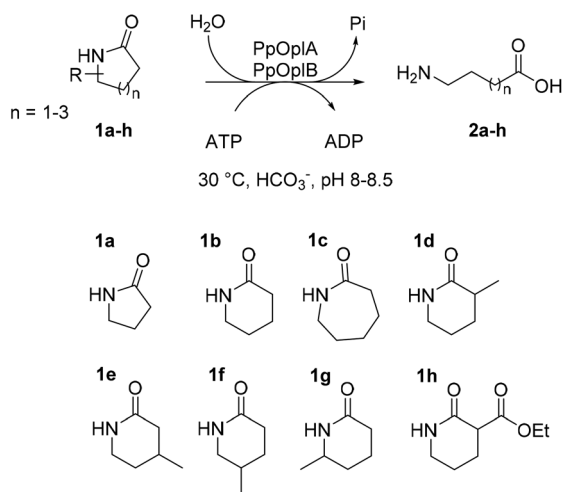


Fig. 2 Enzymatic hydrolysis of medium-sized lactams to the corresponding ω -amino acids by the pair of ATP-dependent oxoprolinases OplA and OplB from *Pseudomonas putida* KT2440 and overview of investigated lactams **1a–h**.

broth under isopropyl β -D-1-thiogalactopyranoside (IPTG) induction at 30 °C resulted in poor soluble expression, as was the case in trials at lower temperatures (20 °C, data not shown), despite good overall over-expression (see ESI, Fig. S1†). The expression was thus performed under autoinduction conditions. The presence of ammonium bicarbonate in the lysis buffer during cell lysis by sonication was crucial for the release of soluble protein (see ESI, Fig. S2 and S3†), especially in the case of PpOplA, while in general PpOplB showed low soluble expression or low release as soluble protein. Due to the very low amounts of PpOplB obtained after purification by immobilized metal affinity chromatography (IMAC, see ESI, Fig. S4†), biotransformations were first performed by combining both cell-free extracts (CFEs) obtained by collecting both supernatants upon cell lysis by sonication.

The first tests were run on 2 mM δ -valerolactam (**1b**) in aqueous ammonium bicarbonate buffer (50 mM, pH 8.5) at 30 °C for 16 h in the presence of 1 eq. ATP and equal amounts of total protein in CFEs of PpOplA and PpOplB. The formation of 5-aminovaleric acid (**2b**) was monitored by gas chromatography after derivatization with ethyl chloroformate (see ESI, Fig. S8–S16†), and reached 1.4 mM (70% conversion), indicating strong activity of the pair PpOplA/PpOplB in the hydrolysis of **1b** (Table 1, entry 2). Full conversion could be obtained in the presence of excess of ATP (2.5 eq., Table 1, entry 3). Under these conditions, no product formation could be detected with γ -butyrolactam (**1a**). The use of a large excess of ATP was found detrimental to the reaction with **1b** as conversion dropped to 35% with 12.5 mM of ATP. Up to 80% conversion could be reached on 5 mM **1b** with 7.5 mM ATP (Table 1, entry 6). Under the tested conditions, no conversion was observed on 10 mM **1b**. Conversions on ϵ -caprolactam (**1c**) remained low at both 2 mM and 5 mM substrate concentration (max. 25%, Table 1, entries 9 and 10), highlighting the preference of the pair PpOplA/PpOplB for the 6-membered ring lactam.

Table 1 Hydrolysis of **1a–c** to the corresponding amino acids **2a–c** by CFEs of PpOplA and PpOplB expressed individually in *E. coli*^a

Entry	Substrate	[1a–c] (mM)	[ATP] (mM) (eq.)	[2a–c] formed (mM)	Conv. to 2a–c (%)
1	1a	2	5 (2.5)	n.d.	n.a.
2	1b	2	2 (1)	1.4	70
3	1b	2	5 (2.5)	~2	>99
4	1b	2	12.5 (6.25)	0.7	35
5	1b	5	5 (1)	2.1	42
6	1b	5	7.5 (1.5)	4.0	80
7	1b	5	10 (2)	3.1	62
8	1b	10	25 (2.5)	n.d.	n.a.
9	1c	2	5 (2.5)	0.5	25
10	1c	5	10 (2)	0.8	16

n.d.: not detected, n.a.: not applicable. ^a Reaction conditions: ammonium bicarbonate buffer (50 mM, pH 8.5) containing MgCl_2 (4 mM), CFEs of PpOplA and PpOplB (11.4 mg mL^{-1} each, total protein content), [ATP] as indicated, [**1a–c**] as indicated, incubation at 30 °C for 16 h and 140 rpm. Reactions performed in duplicates and analyzed by gas chromatography after derivatization of the amino acid and use of a calibration curve with reference materials.

Different control reactions in the absence of ATP, PpOplA, PpOplB, or both PpOplA and PpOplB, were performed in parallel; no amino acid could be detected in any of these cases. No product could be detected when the reaction was run in potassium phosphate buffer (50 mM, pH 7.5) in absence of ammonium bicarbonate, confirming past observations with homologous PjCapAB that bicarbonate is crucial for activity.¹⁹ Noteworthy is that activity was strongly diminished upon freezing and thawing of the enzyme preparations, therefore CFEs were prepared fresh before each round of biotransformation. A mixture of lyophilized whole cells separately expressing PpOplA and PpOplB was found active in the hydrolysis of 2 mM **1b** with 5 mM ATP, however lower conversions were achieved (max. 50% conversion, data not shown). The limitation in using a mixture of whole cells is likely due to the necessary transfer from the first intermediate obtained with PpOplA to the second biocatalyst preparation, which requires exchange from one cell to another. The use of purified proteins was impaired due to the low amounts of PpOplB retrieved (see ESI, Fig. S4†) and first attempts did not lead to any conversion (data not shown). Early studies already indicated the tendency of the purified fragment B from *Pseudomonas putida* to aggregate,²⁵ affecting activity. Given the negative impact of high concentrations of ATP on conversion (see Table 1), a stepwise addition of ATP was implemented. Addition of three batches of 5 mM ATP over 2 h led to a great increase in the formation of **2b** (up to >99% conversion from 6 mM **1b**, and 6.9 mM **2b** obtained from 8 mM **1b**, Table 2, entries 1 and 2). On **1c**, the effects remained modest (up to 1.4 mM **2c**, Table 2, entries 4–6), confirming the preference of the PpOplAB system for the valerolactam ring under these reaction conditions.

Co-expression of PpOplA and PpOplB in the pETDuet1 vector

Given the poor stability of the enzyme preparations upon storage and the low soluble expression of PpOplB (*vide supra*),



Table 2 Stepwise addition of ATP in the hydrolysis of **1b–c** by CFEs of PpOplA and PpOplB expressed individually^a

Entry	Substrate	[1a–c] (mM)	[2a–b] formed (mM)	Conv. to 2a–b (%)
1	1b	6	~6	>99
2	1b	8	6.9	86
3	1b	10	6.7	67
4	1c	6	1.4	24
5	1c	8	1.2	15
6	1c	10	1.2	12

^a Reaction conditions: ammonium bicarbonate buffer (50 mM, pH 8.5) containing MgCl₂ (4 mM), CFEs of PpOplA and PpOplB (11.4 mg mL⁻¹ each, total protein content), ATP (15 mM final concentration, 5 mM added at 0 h, 1 h and 2 h), [**1b–c**] as indicated, incubation at 30 °C for 16 h at 140 rpm. Reactions performed in duplicates and analyzed by gas chromatography after derivatization of the amino acid and use of a calibration curve with reference materials.

improvements in the protein expression were necessary for further investigations of the catalytic activity. We decided to co-express PpOplA and PpOplB using a co-expression vector, taking into account that the crystal structure of the homologous caprolactamase system from *Pseudomonas jessenii* revealed a heterotetrameric assembly, in which each CapB subunit interacts with a CapA subunit. We anticipated that co-expression of both subunits of the OplAB system within the same cell would favor the formation of the tetrameric assembly upon protein expression, likely impacting positively the stability of the whole assembly. In addition, such protocol grandly simplifies the preparation of the biocatalyst. The use of the pETDuet1 vector with two multiple cloning sites allowed successful co-expression of both enzymes in *E. coli* BL21 (DE3) under autoinduction in terrific broth at 24 °C. Noteworthy was the significant increase in the soluble expression of PpOplB and the comparable yields of soluble protein between PpOplA and PpOplB (Fig. 3). This method allowed the generation of a

stable assembly in solution, as demonstrated by the successful co-purification of both proteins by IMAC. Since only PpOplA bears a His-tag at the N-terminus, this points out strong interactions between the two subunits and the formation of a protein complex.²⁶ A 1 : 1 molar yield of the soluble assembly was confirmed by densitometric analysis of SDS-PAGE (see Fig. 3 and ESI, Table S1†). The presence of bicarbonate in the lysis buffer contributed to higher protein yields, but was found in this case not to be mandatory for good release of soluble proteins, which was also obtained in Tris and HEPES buffers for both proteins (see ESI, Fig. S5†).

The activity of co-expressed PpOplA and PpOplB (now described as PpOplAB) was investigated on a range of lactams (Fig. 2). To simplify the analysis and avoid time consuming derivatization for GC analysis, a method was developed that allowed for co-analysis of substrate and product by LC-MS, directly from the aqueous reaction mixture. Calibration curves were generated for all reference amino acids (see ESI, Fig. S17–S22†). PpOplAB was used as CFEs on 10 mM **1a–h** in the presence of 12.5 mM ATP (total protein content of 8 mg mL⁻¹, corresponding to ~7 μM of PpOplAB based on densitometric analysis, see Table S1†). Strongest activity could be observed on **1b** (full consumption) and 5-methylpiperidin-2-one (**1f**, leading to 7.9 mM product, Table 3), while conversion on **1c** remained modest (2.3 mM **2c** formed, Table 3, entry 3). For the first time, some product could be observed in the conversion of γ-butyrolactam (**1a**), corresponding to ~4% substrate consumption (see ESI, Fig. S26†). With other methyl derivatives of δ-valerolactam, conversions were moderate (max. 2.4 mM product, Table 3) and substitution closest to the amide bond (especially 3- and 6-position) impacted the conversion negatively the most. In contrast, substitution furthest to the carbonyl had almost no impact on activity, pointing at possible steric hindrance near the reactive moiety with **1d**, **1e** and **1g**. Finally, 3-ethoxycarbonyl-2-piperidone (**1h**) with a large and electron-withdrawing substituent in α-position to the carbonyl was not accepted (data not shown). All substituted

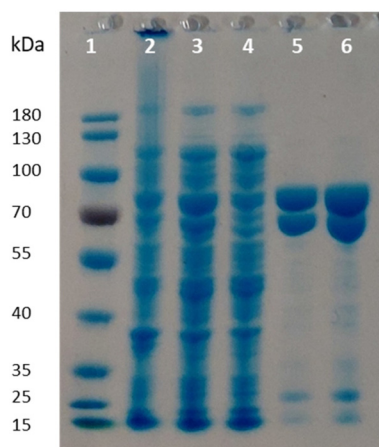


Fig. 3 SDS-PAGE following co-expression of PpOplA and PpOplB using the pETDuet-1 vector (MW PpOplA including His-tag: ~76 kDa, MW PpOplB: ~63 kDa). Lane 1: PageRuler™ Prestained Protein Ladder; lane 2: pellet; lane 3: supernatant (soluble fraction); lane 4: flow through from IMAC; lanes 5 and 6: desalted purified enzyme fraction.

Table 3 Hydrolysis of lactams **1a–h** with co-expressed PpOplAB^a

Entry	Substrate	[2a–h] (mM)	Conv. to 2a–h (%)
1	1a	n.d. ^b	~4
2	1b	7.7 (±0.8)	77 ^c
3	1c	2.3 (±0.1)	23
4	1d	1.3 (±0.1)	13
5	1e^d	2.4 (±0.1)	24
6	1f	7.9 (±0.3)	79
7	1g	1.4 (±0.0)	14
8	1h	n.f.	n.a.

^a Reaction conditions: 8 mg mL⁻¹ PpOplAB CFE (total protein content), 10 mM **1a–h**, 12.5 mM ATP, ammonium bicarbonate buffer (39 mM, pH 8.5), 8 mM MgCl₂, 30 °C, 900 rpm, 24 h. Reactions performed in triplicates and analyzed by LC-MS (standard deviation indicated). ^b n.d.: not determined, product formation not quantified. ^c 4% substrate consumption (see ESI, Fig. S26†). ^d n.f.: not formed, n.a.: not applicable. ^e Substrate fully converted. See ESI, Fig. S26–S33.† ^d E-Value of 6, see text and ESI.†



The ATP concentration was found to correlate well with the amount of product formed, and in general, some uncoupling can be anticipated, as the amount of formed **2b** was found inferior to the ATP concentration used in all cases (see

ATP is required in the reaction of OplAB on 5-oxo-L-proline²⁰ and of CapAB on ϵ -caprolactam.¹⁸ An ATP binding site was detected in PjCapA and relevant amino acids involved in binding are conserved in PpOplA.¹⁹ ATP was proposed to activate the substrate by phosphorylation through the action of subunit A, and is released as ADP, while subunit B hydrolyzes the phosphorylated species in a subsequent step.^{19,20} This explains the requirement for at least stoichiometric amounts of ATP, which is practically not compatible with atom-economic and cost-effective biocatalytic applications. The use of catalytic quantities of ATP for the biocatalytic hydrolysis of lactams was therefore investigated by implementing an enzymatic regeneration system based on polyphosphate kinase 2 from class I (PPK2-I), which catalyzes the monophosphorylation of ADP from cheap inorganic polyphosphate (Fig. 4).²⁷ The PPK2-I SMc02148 from *Sinorhizobium meliloti*²⁸ was selected because this enzyme was shown compatible with other ATP-dependent enzymatic systems.²⁹ The enzyme was over-expressed in *E. coli* (see ESI, Fig. S6†) and added as CFE to the standard reaction mixture supplemented with cheap Graham's salt (*i.e.*, sodium metaphosphate). Full conversion of 10 mM **1b** could be observed in the presence of 0.5 mM ATP, 5 mM Graham's salt and 25 mM ammonium bicarbonate (see Table 5). Without the kinase, the conversion was strictly

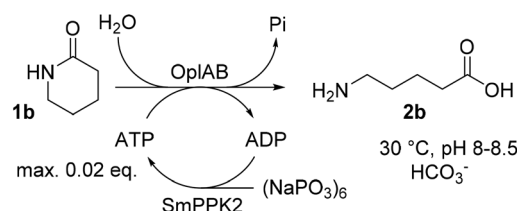


Table 4 Influence of [ATP] on product formation in the PpOplAB-catalyzed hydrolysis of **1b**^a

Entry	[ATP] (mM)	[2b] (mM)	Relative activity (%)
1	Not provided	n.d.	n.a.
2	5	2.7 ± 0.2	36
3	10	5.3 ± 1.4	70
4	12.5	7.6 ± 0.0	100

Entry	Varying conditions	Relative activity (%)
1	Standard ^a	100 ^b
2	No PPK2-I ^c	3
3	20 mM Graham's salt ^c	86
4	No PPK2-I ^c , 12.5 mM ATP (1.25 eq.)	31

¶The *E*-value was computed using the freely accessible program available at <https://biocatalysis.uni-graz.at/biocatalysis-tools/enantio>, with calculations based on work detailed in the literature.³²

limited to the catalytic amount of ATP present (<5% conversion). Higher amount of polyphosphate lowered the conversion. Under the tested reaction conditions, a clear advantage of using the ATP recycling system could be seen, compared to the addition of excess of ATP, which led to only 31% of the activity obtained with the recycling system (see Table 5, entry 4). Further tests were performed, indicating that 0.1 mM ATP was sufficient to reach full consumption of 10 mM **1b** (see Table 6). On 25 mM of **1b**, increasing the ATP concentration from 0.1 mM (0.004 eq.) to 0.5 mM (0.02 eq.) had a strong positive impact on conversion and up to 13.9 mM of **2b** was formed in the presence of 10 mM Graham's salt (Table 6). A time study indicated that the reaction was particularly rapid during the first 90 min (7.9 mM of **2b** formed) and then began to slow down, with maximum conversion reached after about 10 h (see ESI, Fig. S7†). Increasing the substrate concentration to 50 mM barely improved the product formation (14.7 mM of **2b**), even in the presence of higher bicarbonate concentration. Collectively, the data indicate that the PpOplAB/SmPPK2 platform can generally sustain high substrate concentrations. Based on densitometric analysis of SDS-PAGE, the amounts of enzyme in the crude lysate were estimated for PpOplAB (3.4 μ M) and SmPPK2 (18.1 μ M). In the case of the biotransformation of 50 mM **1b** that led to the formation of 14.7 mM **2b**, PpOplAB achieved a turnover number (TON) of 4360 and SmPPK2, a TON of 785 (see ESI, Table S1†). Both values indicate a good to excellent catalytic efficiency in the cleavage of δ -valerolactam under ATP-regeneration conditions.

Similarly to PjCapAB, PpOplAB requires bicarbonate for activity. A direct correlation was seen between the concentration of bicarbonate and the amount of product formed, with highest product concentration obtained in the presence of excess of bicarbonate (*vide supra*, and see ESI, Table S2†). With PjCapAB, a carboxyphosphate formed from bicarbonate and ATP was postulated as a possible intermediate able to phosphorylate the substrate, among various mechanistic proposals.¹⁹ We therefore hypothesized that other phosphodonors similar to carboxyphosphate could bypass the strict require-

ment for ATP. A range of alternative phosphodonors (Fig. 5) was explored and added in place of excess of ATP (1.25 eq.). With 2-phosphoenolpyruvate (**3c**), no product formation could be detected. In contrast, the use of both carbamoyl phosphate (**3a**) and acetyl phosphate (**3b**), structurally related to carboxyphosphate **3d**, resulted in the formation of 0.3 mM **2b**, corresponding to 10% of the activity obtained with the same amounts of ATP (Table 7). PpOplAB is thus catalytically active in the hydrolysis of **1b** in absence of ATP and achieved a TON of 176 with the alternative phosphodonors **3a** and **3b**. These data provide some clues that the structurally related carboxyphosphate may be involved in the first step of the reaction and that under standard conditions, ATP may not be directly phosphorylating the substrate, contrarily to what was postulated for one bacterial 5-oxoprolinase, for which no bicarbonate dependency was reported.^{20,25} This would suggest a role of ATP with PpOplAB similar to the cases of acetone and acetophenone carboxylases, which generate carboxyphosphate as a first intermediate in the catalytic cycle. These enzymes however transfer the carboxy moiety, and not the phosphate, to their respective substrate. The phosphotransfer to the substrate by PpOplAB may not be enzymatic and cases of spontaneous phosphorylation of a range of acceptors with acetyl phosphate have been reported.³⁰ Unambiguous is that PpOplA and other homologous subunits are necessary to allow the utilization of ATP as primary source of phosphate *en route* to substrate activation, which exact mechanism remains to be elucidated.

³¹P-NMR

Given that structures similar to carboxyphosphate can trigger hydrolysis of the lactam by PpOplAB in the absence of ATP, it appears likely that the role of ATP is to generate a phosphorylated intermediate species for activation of the substrate. While phosphotransfer from carboxyphosphate to the sub-

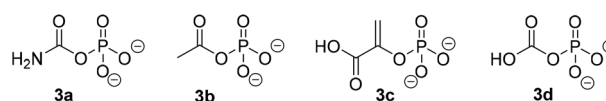


Fig. 5 Phosphodonors (**3a**–**3c**) tested in place of ATP with PpOplAB and structure of carboxyphosphate **3d**.

Table 6 Conversion of **1b** to **2b** by PpOplAB with ATP regeneration^a

Entry	[1b] (mM)	[ATP] (mM) (eq.)	[2b] formed (mM)
1	10	0.1 (0.01)	7.8 $\pm$ 0.4 ^b
2	10	0.5 (0.05)	7.4 $\pm$ 0.1 ^b
3	25	0.1 (0.004)	7.9 $\pm$ 1.7
4	25	0.5 (0.02)	13.9 $\pm$ 3.0
5	50	0.1 (0.002)	11.4 $\pm$ 1.0
6	50 ^c	0.5 (0.01)	14.7 $\pm$ 1.1

^a Reaction conditions: 4 mg mL⁻¹ PpOplAB CFE (total protein content), 5 mg mL⁻¹ SmPPK2 CFE (total protein content), [**1b**] as indicated, [ATP] as indicated, 10 mM Graham's salt (equivalent to 60 mM monophosphate), 1 vol% glycerol, 10 mM NaCl in ammonium bicarbonate buffer (pH 8.5). Final concentration MgCl₂: 7.3–9.2 mM and ammonium bicarbonate: 27–37 mM (variations due to different levels of dilution after addition of stock solutions of **1b** and ATP in water), 900 rpm, 30 °C, 24 h. Mean of triplicates with standard deviation.

^b Full consumption. ^c Increasing [NH₄HCO₃] to 25 mM did not improve the product formation (data not shown).

Table 7 Influence of the type of phosphodonor in the PpOplAB-catalyzed hydrolysis of **1b**^a

Entry	Phosphodonor	[2b] formed (mM)	Relative activity (%)
1	3a	0.3 $\pm$ 0.00	10
2	3b	0.3 $\pm$ 0.01	10
3	3c	n.d.	n.a.
4	ATP	2.9 $\pm$ 0.2	100

n.d.: not detected, n.a.: not applicable. ^a Reaction conditions: 2 mg mL⁻¹ PpOplAB CFE (total protein content), 10 mM **1b**, 12.5 mM phosphate donor, 5 mM ammonium bicarbonate in HEPES buffer (50 mM, pH 8.0, 7.8 mM MgCl₂), 30 °C, 900 rpm, 20 h. Mean of triplicates with standard deviation. See ESI, Fig. S35.†



Table 8 Correlation between hydrolysis of ATP to ADP and hydrolysis of **1b** to **2b**^a

Entry	Conditions	[2b] ^b (mM)	ATP/ADP ^c	[ADP] ^d (mM)	Uncoupling ^e (%)
1	10 mM 1b	2.0	1 : 2.28	8.7	77
2	No substrate	n.a.	1 : 0.60	4.7	37

n.a.: not applicable. ^a Reaction conditions: 1 mg mL⁻¹ PpOplA/B CFE (total protein content), 12.5 mM ATP, ammonium bicarbonate buffer (39 mM, pH 8.5), 8 mM MgCl₂, 30 °C, 900 rpm, 1 h. ^b Amount of formed **2b** quantified by HPLC-MS. ^c Ratio obtained by ³¹P-NMR analysis. ^d Obtained based on the measured ratio of ATP/ADP. ^e Corresponds to the percentage of ADP formed by ATP hydrolysis and not by phosphorylation of **1b**, value obtained by $[\text{ADP}] - [\text{2b}]/[\text{ADP}] \times 100$.

strate has been proposed to happen, a carboxylation of the oxygen of the tautomeric form of the lactam, the lactim, to a carbonate cannot be ruled out. This would go in line with the carboxylating role of carboxyphosphate in a number of enzymatic reactions.^{21–23} The resulting carbonate monoester would then be hydrolyzed by PpOplB. Further investigations of the mechanism were attempted by ³¹P-NMR, with the aim to reveal the formation of phosphorylated intermediate species. The reaction mixture of the biotransformation of **1b** in the presence of excess of ATP (12.5 mM) under conditions similar to those reported in Table 3 was analyzed in parallel by LC-MS. ³¹P-NMR allows to discriminate between most phosphorylated species, especially when different numbers of phosphate units are involved, and can be used to easily monitor the conversion of ATP to ADP. After 1 h reaction catalyzed by PpOplAB, 2 mM of **2b** was formed and a ratio of ATP/ADP of 1 : 2.28 was measured (see Table 8 and ESI, Fig. S40 and S41†), indicating 70% hydrolysis of ATP, which corresponds to 8.7 mM of ADP formed. Taking into consideration the formation of 2.0 mM of product, the uncoupling of ATP therefore reaches 77%. This highlights the importance of excess of ATP to achieve high conversion levels. Noteworthy is that ATP could be hydrolyzed to ADP in absence of lactam (see Table 8, entry 2). This goes in line with previous observations with PjCapAB, which catalyzes futile hydrolysis of ATP.¹⁹ A higher ATP hydrolysis compared to the amount of glutamate formed was also observed in early studies on bacterial 5-oxo-L-prolinase.²⁰ In the case of the *N*-methylhydantoinase system from *Pseudomonas putida* 77,³¹ γ -butyrolactam and δ -valerolactam could trigger the consumption of ATP, however hydrolysis to the amino acid did not take place, indicating that substrate selectivity is very much subunit and enzyme dependent.

Finally, during our studies, ³¹P-NMR analysis did not allow to discriminate between potential further phosphorylated intermediates during the reaction.

Experimental

Procedures for biotransformations

Representative biotransformations using CFEs of PpOplA and PpOplB expressed individually. The cell pellets of PpOplA

and PpOplB obtained by individual overexpression were resuspended separately in lysis buffer (ammonium bicarbonate, 50 mM, pH 8.5 containing 4 mM MgCl₂) and disrupted by sonication. The cell debris were removed by centrifugation (17 000 rpm, 30 min, 4 °C) and the protein concentration of PpOplA and PpOplB was measured in the CFEs by the Bradford assay. Reactions were performed in 2 mL micro reaction tubes in ammonium bicarbonate buffer (50 mM, pH 8.5) containing MgCl₂ (4 mM), CFEs of PpOplA and PpOplB (11.4 mg mL⁻¹ each, total protein content), ATP and lactam in concentrations as indicated, in a final volume of 1 mL. The samples were incubated with horizontal shaking for 16 h at 30 °C and 140 rpm. Two control reactions were run in parallel in the absence of (i) enzyme and (ii) substrate. After this time, the mixtures were filtered by centrifugation and the supernatant was split into two 2 mL micro reaction tubes (each 450 μ L). One sample was lyophilized to be used for amino acid derivatization and further measurements, and the other tube was used for lactam extraction, as reported in the ESI.†

Representative biotransformations using CFEs of co-expressed PpOplAB and ATP-regeneration system with a kinase. The cell pellets containing PpOplAB were resuspended in 6 mL ammonium bicarbonate buffer (50 mM, pH 8.5, 10 mM MgCl₂) per g cell mass, the cells were disrupted by sonication and cell debris were removed by centrifugation (18 000 rpm, 30 min, 4 °C) to deliver CFEs. Biotransformations were performed on an analytical scale (500 μ L if not stated otherwise) in 1.5 mL micro reaction tubes. The reactions were run in ammonium bicarbonate buffer (pH 8.5, 27–37 mM) containing 10 mM NaCl and MgCl₂ (final concentration 7.3–9.2 mM), 4 mg mL⁻¹ PpOplAB CFE (total protein content), 5 mg mL⁻¹ SmPPK2 CFE (total protein content), lactam and ATP in concentrations as indicated, 10 mM Graham's salt (equivalent to 60 mM monophosphate), 1 vol% glycerol. The samples were incubated in an Eppendorf Thermomixer at 900 rpm, 30 °C, 24 h. 25 μ L reaction volume were used for analysis by LC-MS, as detailed in the ESI.†

Conclusions

The chemical stability of δ -valerolactams could be efficiently circumvented and enzymatic hydrolysis to the ω -amino acids proceeded under mild reaction conditions in aqueous medium at the expense of ATP in presence of bicarbonate. The bacterial system PpOplAB likely utilizes the lactim tautomer of the lactam as reactive species, taking advantage of the significant partial C–N double bond character of the amide bond responsible for the resonance stabilization of the δ -lactams. A kinase-based ATP regeneration with inexpensive Graham's salt was successfully implemented, and is especially advantageous given the substantial ATP uncoupling observed during lactam hydrolysis, which leads to futile hydrolysis of ATP. Although the intermediate species involved in the reaction were not identified, the fact that ATP could be replaced by the alternative phosphodonors carbamoyl phosphate and acetyl phos-



phate provides hints that the role of bicarbonate in the reaction is to generate a carboxyphosphate intermediate for substrate activation. PpOplAB is not effective in the hydrolysis of γ -butyrolactam. However, the trace activity observed is promising and suggests that the chemical stability of this molecule may be tackled in the future using biological tools.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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