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ATP regeneration system in chemoenzymatic amide bond formation with thermophilic CoA ligase

Chloé M. Lelièvre, Mélanie Balandras, Jean-Louis Petit, Carine Vergne-Vaxelaire* and Anne Zaparucha* [a]

Abstract: CoA ligases are enzymes catalyzing the ATP-dependent addition of coenzyme A to carboxylic acids in two steps through an adenylate intermediate. This intermediate can be diverted by a nucleophilic non enzymatic addition of amine to get the corresponding amide for synthetic purposes. To this end, we selected thermophilic CoA ligases to study the conversion of various carboxylic acids into their amide counterparts. To limit the use of ATP, we implemented an ATP regeneration system combining polyphosphate kinase 2 (PPK2 Class III) and inorganic pyrophosphatase. Suitability of this system was illustrated by the lab-scale chemoenzymatic synthesis of *N*-methylbutyrylamide in 77% yield using low enzyme loading and 5% molar ATP.

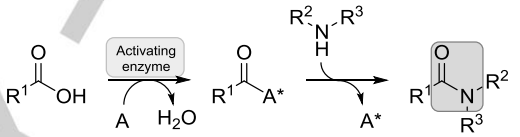
Introduction

Amide function is widespread in many natural and synthetic compounds such as active pharmaceutical ingredients (API) and polymers.[1] The amide bond formation is therefore essential for chemical industries.[2] As an example, *N*-acylation to amide represents about 16% of overall transformations in medicinal reactions.[3]

Amide bond synthesis consists in the condensation of carboxylic acid and amine. The most common strategy is the activation of the acid by coupling reagents before nucleophilic amine addition.[4] In conventional chemistry, popular reagents used for activating acid are carbodiimides, oxalyl and thionyl chlorides along with 1,1-carbonyldiimidazole (CDI).[5] However, these processes result in poor atom economy and lead to the production of hazardous by-products. In 2007, 'amide bond formation avoiding poor atom economy reagents' was assigned as the research area with highest priority by the ACS Green chemistry institute and global pharmaceutical companies.[6]

Enzymatic catalysis may eliminate many of the issues caused by organic coupling reagents.[7] In nature, most of the amide bonds are formed in complex enzyme machineries such as ribosomes, using adenosyl triphosphate-dependent aminoacyl-tRNA synthetases, and nonribosomal peptide synthetases (NRPS).[8] At an industrial scale, biocatalytic approaches starting from carboxylic acids are rare, the main example being the synthesis of β-lactams by penicillin G acylases.[9] From other substrates, we can notice the use of nitrile hydratases for the production of acrylamide, esterases, lipases and proteases for biocatalytic aminolysis and transglutaminases for the modification of Gln-containing peptides.[10] However, these enzymes often have a limited substrate range and/or require non aqueous reaction conditions.[7b]

In the last past years, biocatalytic amide bond formation from activated carboxylic acids has been the subject of a growing number of publications reporting the use of NRPS stand-alone adenylation domain (SAAD), carboxylate reductase adenylation domain-plus-peptidyl carrier proteins (CAR-A-PCP), amide-bond synthetases (ABS), coenzyme A (CoA) ligases and AMP-ligating enzyme (Scheme 1).[11] In all these reported biocatalytic synthesis, the activating agent is used as equivalent ratio.



Scheme 1. Biocatalytic carboxylic acid activation reaction for the amide bond formation.

CoA ligases are members of the adenylate-forming enzyme superfamily (ANL) which includes firefly luciferase, various acyl-CoA synthetases and adenylation domains of NRPS.[12] These enzymes are involved in fatty acid metabolism where the acyl-adenylate intermediate leads to the CoA-thioester by displacement of the adenylate group by the nucleophilic CoA. Several bacterial CoA ligases have been reported ranging from short chain acid CoA ligases to fatty acid CoA ligases.[13] With the goal to implement a short and efficient process for amide bond formation with synthetic purposes, we decided to build a chemoenzymatic cascade involving this CoA ligase family for its enzymatic ability to activate carboxylic acids. By coupling them with amines in a non-catalyzed step, this can give rise to various amides without any amine substrate scope restriction except for their nucleophilicity. CoA ligases catalyze the formation of CoA thioesters from carboxylic acids in two steps. For a more valuable process, we decided to divert the reaction from the adenylate intermediate by performing the non-catalyzed amine addition directly without going up to the CoA thioester. This would allow the elimination of the use of the costly CoA. To promote the nucleophilic attack of the amine, one solution is to work at high temperatures. We therefore focused on thermophilic CoA ligases.

We herein report the chemoenzymatic amide bond formation with a thermophilic CoA ligase from *Metallosphaera sedula* which catalyzes the activation of various carboxylic acids at high temperature, together with the lab-scale synthesis of *N*-methylbutyrylamide as an example of this approach.[14] To

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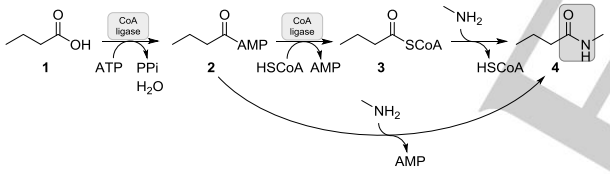
improve the suitability of such system, we implemented an adenosyl triphosphate (ATP) regeneration system with a thermophilic polyphosphate kinase 2 Class III from *Deinococcus geothermalis* catalyzing the phosphorylation of both nucleoside mono- and diphosphate and a thermophilic inorganic pyrophosphatase from *Pyrococcus horikoshii* OT3 hydrolyzing pyrophosphate into inorganic phosphate to eliminate pyrophosphate inhibition.[15]

Results and Discussion

We decided to set up the mediated CoA-ligase thermal chemoenzymatic synthesis of amides derived from butyric acid as model reaction. We first selected the thermophilic CoA ligase Msed_0406 from *Metallosphaera sedula* (UniprotKB ID: A4YDT1 renammed here *Msed*CoAlig$_1$,) reported to be active towards a broad range of small substrates at high temperature.[14]

This enzyme showed a temperature activity profile with an optimum at 75°C (Figure S1). First of all, we tested the conversion of butyric acid into amides with various amines, including amino acids, in the presence of CoA (see experimental section and Table S1 for details). As expected, we observed the formation of the corresponding amides for all of them, which validates the versatility of the approach (Figures S16-S25).

We next studied the formation of the amide product directly from the adenylate intermediate to avoid the use of the expensive reactant CoA (Scheme 2).



Scheme 2. Chemoenzymatic synthesis of *N*-methylbutyrylamide (**4**) with CoA ligase.

To this end, we compared three chemoenzymatic reaction systems with methylamine as amine nucleophile (5 or 50 molar equivalents): CoA ligase with CoA as standard reaction, CoA ligase with *N*-acetylcysteamine (Nac) as CoA mimic and CoA ligase without any thiol derivative.[16] The reactions were carried out at 50°C, 65°C and 80°C. The formation of the *N*-methylbutyrylamide (**4**) product was monitored by UHPLC-MS. (Figure 1).

Figure 1. Effect of presence of CoA on *N*-methylbutyrylamide (**4**) formation at different temperatures with 5 or 50 molar equivalents of methylamine. Reaction conditions: 50 mM phosphate buffer pH 8.0, 5 mM **1**, 5 mM $MgCl_2$, 0.1 mg/mL *Msed*CoAlig$_1$, 5 mM ATP with 5 mM CoA or Nac, 7 h. Error bars represent the s.d. of two independent experiments.

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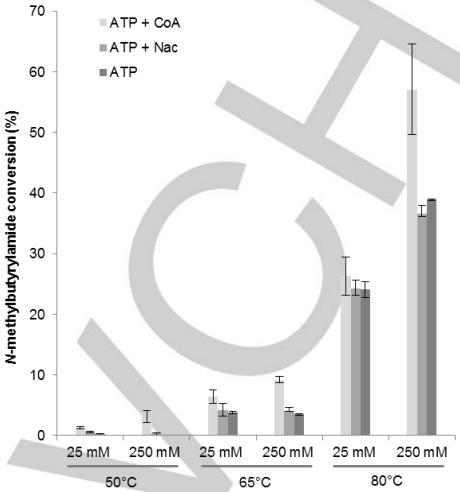
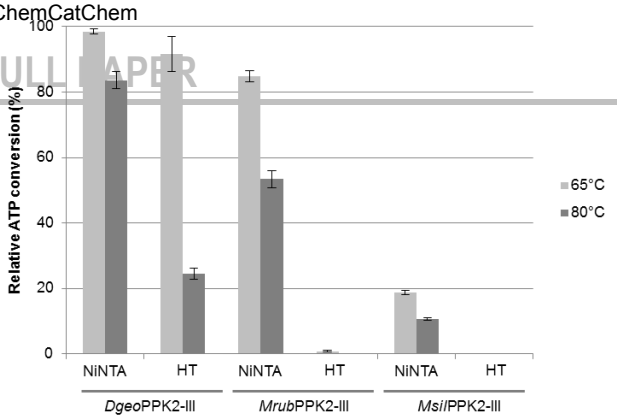


As expected, amide formation was observed without CoA in the reaction media, indicating that the nucleophilic amine addition can occur on the adenylate intermediate. Increase of temperature and molar equivalents of methylamine led to better conversions, since the nucleophilic attack is favored and the enzyme activity is enhanced. In these conditions, 65°C seems to be the minimal working temperature to get more than traces of product. Although the formation of amide **4** is favored in presence of CoA, in its absence, conversions observed at 65-80°C were found to be sufficient to continue with a CoA-free system. Results obtained with Nac were similar to those in absence of CoA thus hypothesizing that Nac is not accepted as co-substrate by this enzyme.

We next turned to the implementation of an enzyme-based regeneration system for ATP. Rather than setting up an ATP regeneration system combining two PolyPhosphate Kinases 2, PPK2-II and PPK2-I, catalyzing respectively the phosphorylation of AMP to ADP and ADP to ATP, we chose PPK2 Class III for its capability to phosphorylate both AMP to ADP and ADP to ATP, or to add pyrophosphate to AMP, with the inexpensive polyphosphate as phosphate source.[17] To fit the reaction conditions, three thermophilic PPK2-III were selected: *Dgeo*PPK2-III (UniprotKB ID Q1IW43) from *Deinococcus geothermalis*, *Mrub*PPK2-III (UniprotKB ID M9XB82) from *Meiothermus ruber* and *Msil*PPK2-III (UniprotKB ID D7BBL3) from *Meiothermus silvanus*.[15b] The three enzymes were overexpressed and purified by common nickel affinity chromatography or by heat treatment. The reactions were carried out at 65°C and 80°C. ATP formation was monitored by HPLC-UV (Figure 2).

Figure 2. AMP to ATP conversions with the three purified PPK2-III at 65 and 80°C (NiNTA: purification by nickel affinity chromatography, HT: purification by heat treatment). Reaction conditions: 50 mM MOPS buffer pH 8.0, 0.5 mM AMP, 20 mM $MnCl_2$, 10 mM $PolyP_6$, 0.01 mg/mL PPK2-III, 30 min. For more details, see Experimental Section.



For the three enzymes, higher conversions were obtained at the reported optimal temperature of 65°C.[15b] We selected *Dgeo*PPK2-III which gave the best conversions (> 80%) while being easily purified by heat treatment with maintained activity at 65°C. Based on reported experimental conditions for both selected enzymes (*Msed*CoAlig$_1$ and *Dgeo*PPK2-III), the effect of buffer and cations on the conversion rate in *N*-methylbutyrylamide (**4**) was studied in an ATP regeneration-free system. MOPS buffer pH 8.0 in presence of limited amount of $MnCl_2$ (5 mM), was preferably chosen to the initial phosphate buffer and $MgCl_2$ (Table S2).

It has been previously reported that pyrophosphate (PPi) could be an inhibitor of CoA ligase and that this inhibition can be lifted by addition of inorganic pyrophosphatase (PPase) which catalyzes the hydrolysis of PPi into inorganic phosphate (Pi).[18] We confirmed that in our reaction system, *Msed*CoAlig$_1$ was inhibited by PPi in a non-competitive way (Figure S2). To refine our ATP regeneration system, we therefore selected PPases able to tolerate high temperatures and tested them in our reaction. Among the five thermophilic PPases tested, all showed similar activity profiles with total hydrolysis of 1 mM PPi at 65-70°C in presence of $MnCl_2$, 5 mM in MOPS buffer 50 mM (maximum pH 8.5) after 10 min with 0.02 mg.mL^{-1} of enzyme (Table S3). The one from *Pyrococcus horikoshii* OT3 *Ph*PPase (UniprotKB ID O59570) was chosen for its easy overexpression in *E. Coli* and purification but also for its stability at 65°C (no loss of activity after 17 h).[15a] Investigation of effects of length and molar Pi equivalent of polyphosphate (PolyP$_n$) led to a maximum of conversion with 2.5 Pi molar equivalents which represent 4.2 mM PolyP$_6$ or 1 mM PolyP$_{25}$ (Figure 3).

The 1 mM PolyP$_{25}$, hypothesized to be the good balance between amount of polyphosphate available for the kinase and its solubility in the buffered system, was kept for further optimization studies. We noticed that the addition of *Ph*PPase into the chemoenzymatic reaction with ATP regeneration system, led to 1.6-3.2 fold-improved conversion of butyric acid (**1**) into *N*-methylbutyrylamide (**4**), in the case of excess of PolyP$_n$. After further optimization works, particularly $MnCl_2$ concentration and buffer pH (SI, figures S3 and S4), the implemented chemomultienzymatic system enabled the 42% conversion of acid **1** (5 mM initial concentration) into amide **4** in 2 h and 88% in 24 h. With these reaction conditions, we investigated the same transformation with other thermophilic CoA ligases also reported to be active towards small carboxylic acids: Msed_0394 (UniprotKB ID A4YDR9) from the same organism than *Msed*CoAlig$_1$, FacI2 (UniprotKB ID A4INB3) from *Geobacillus thermodenitrificans* and a CoA ligase from *Sulfolobus tokodaii* (UniprotKB ID Q973W5) renamed respectively as *Msed*CoAlig$_2$, *Gthe*CoAlig and *Stok*CoAlig (Figure 4).[13a,14, 19] The four enzymes enable the conversion of carboxylic acid **1** from 55% with *Msed*CoAlig$_2$, to 100%, with *Gthe*CoAlig, over 24 h, compared to 88% with our reference *Msed*CoAlig$_1$. It is worthy to note that a nearly quantitative conversion was obtained with *Gthe*CoAlig in only 2 h.

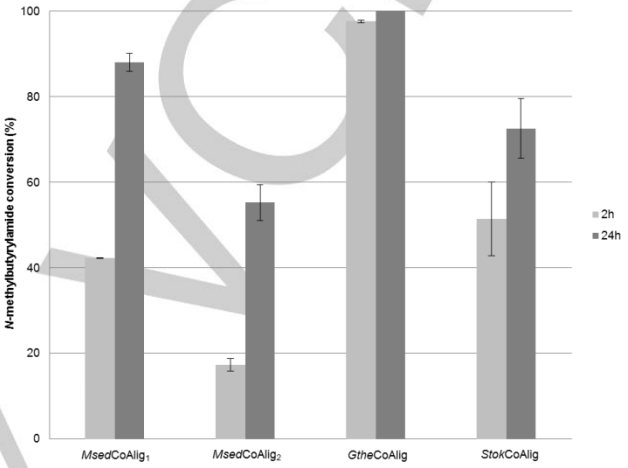


Figure 3. Effects of polyphosphate length and Pi molar equivalent on amide bond formation in presence or absence of PPase. Reaction conditions: 50 mM MOPS buffer pH 8.0, 5 mM **1**, 250 mM $MeNH_2$, 0.5 mM ATP, 5 mM $MnCl_2$, 0.1 mg/mL*Msed*CoAlig$_1$, 0.01 mg/mL*Dgeo*PPK2-III, 0.002 mg/mL*Ph*PPase, 65°C, 24 h.

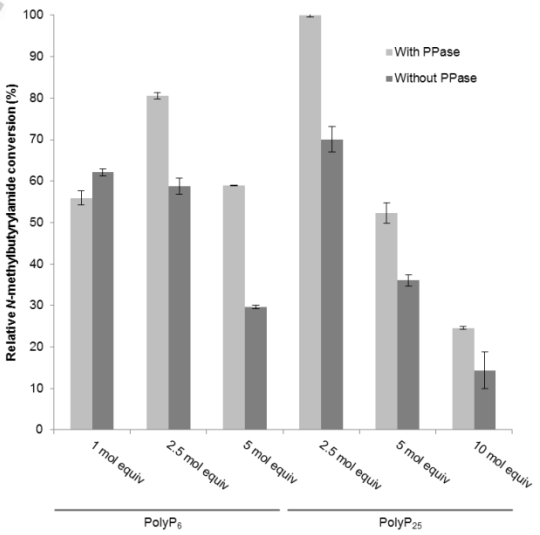


Figure 4. Conversions in *N*-methylbutyrylamide (**4**) with various thermophilic CoA ligases with the ATP-regenerating chemoenzymatic cascade. Reaction conditions: 50 mM MOPS buffer pH 8.5, 5 mM **1**, 250 mM $MeNH_2$, 0.5 mM ATP, 5 mM $MnCl_2$, 1 mM PolyP$_{25}$, 0.1 mg/mLCoA ligase, 0.02 mg/mL*Dgeo*PPK2-III, 0.002 mg/mL*Ph*PPase, 65°C.

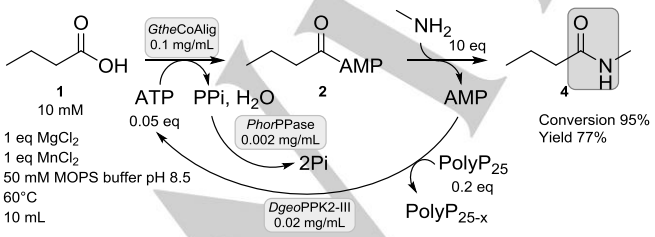
These promising results prompted to explore the substrate scope of the four enzymes, by following the formation of the CoA thioesters from various carboxylic acids by spectrophotometric assay (Table 1). Among them, *Msed*CoAlig$_2$ confirmed its lowest efficiency towards the targeted carboxylic acids. The three others (*Msed*CoAlig$_1$, *Gthe*CoAlig and *Stok*CoAlig) exhibited reasonable specific activities towards short carboxylic acids, up to 1936 - 3272 mU/mg for *Gthe*CoAlig towards non-functionalized short linear carboxylic acids. Interestingly, *Msed*CoAlig$_1$ was found to have the widest substrate scope, being active towards all the tested substrates but with weak specific activities.

Table 1. Specific activities (mU/mg of enzyme) of selected thermophilic CoA ligases towards various carboxylic acids.

	*Msed*CoAlig$_1$	*Msed*CoAlig$_2$	*Gthe*CoAlig	*Stok*CoAlig
Propionic acid	1513.9 ± 377.0	22.8 ± 7.3	1936.5 ± 831.6	212.0 ± 2.5
Butyric acid	943.6 ± 127.0	57.5 ± 12.7	3272.2 ± 839.6	127.9 ± 28.3
Pentanoic acid	761.8 ± 164.5	26.5 ± 7.0	3211.2 ± 1756.1	-
3-phenylpropanoic acid	15.1 ± 2.4	-	-	-
5-oxohexanoic acid	13.6 ± 6.8	232.1 ± 76.9	751.2 ± 197.0	-
Proline	9.9 ± 3.8	-	-	-
Decanoic acid	71.6 ± 28.4	92.2 ± 36.1	-	-

(-): no detected activity. Specific activity of 1 U/mg corresponds to 1 µmol of substrate converted per minute and per mg of enzyme. Error bars represent s.d. of three independent experiments. For more details, see Experimental Section.

A lab-scale reaction with *Gthe*CoAlig has been carried out under optimized conditions with slight higher concentration of butyric acid (10 mM) (Figures S5 and S6) and a reduced amount of methylamine nucleophile (10 molar equivalents, Figure S7). Amide **4** was obtained in 77% crude yield (95% conversion) after 24 h from a 10 mL-scale reaction (TN = 4736) (Scheme 3).



Scheme 3. Lab-scale chemoenzymatic synthesis of *N*-methylbutyrylamide (**4**).

Conclusions

A chemomultienzymatic protocol for the synthesis of amides from short carboxylic acids was implemented. Thanks to a smart ATP-regeneration enzyme-catalyzed step, access of various amides was achieved in presence of thermophilic CoA ligase, benefiting from the carboxylic acid activation capability of CoA ligases and ATP regeneration potential of PolyPhosphate Kinase 2 Class III from AMP. Operating at high temperature avoids the use of the expensive CoA and enhances amide formation. After optimization studies, the potential of such chemoenzymatic cascade was illustrated by the synthesis of *N*-methylbutyrylamide from 10 mM butyric acid. We managed to get 95% conversion with low enzyme loading, 10 molar equivalents of methylamine and only 5% molar ATP. Other carboxylic acids can be used as suggested by the substrate scope performed with four different thermophilic CoA ligases. The non-catalyzed amine attack enables the access to a wide range of amides with only amine nucleophilicity restriction. Further work is underway to expand this protocol to the synthesis of valuable amides. The ATP regeneration system implemented in this synthesis could advantageously be widespread to other biocatalytic processes using AMP-forming enzymes.

Experimental Section

Chemicals and equipment

All reagents were purchased from commercial sources and used without additional purification. PolyP$_6$ was purchased from Sigma Aldrich (MilliporeSigma, StLouis, USA) and polyP$_{25}$ was purchased from Merck (KGaA Darmstadt, Germany).

UHPLC analyses were performed on a UHPLC U3000 RS 1034 bar instrument (Thermo Fischer Scientific) coupled to a mass spectrometer MSQ Plus with electrospray ESI in positive mode for *N*-methylbutyrylamide (**4**) (cone voltage = 50 V) and for *S*-butyryl-*N*-acetylcysteamine (butyrylNac) (cone voltage = 75 V) and negative mode for butyrylCoA (**3**) (cone voltage = 75 V). The probe temperature was set up at 450°C. Spectrophotometric assays were recorded on a Safas UVMC2 (Safas, Monaco) thermostated with a refrigerated/heating circulator Corio CD-200F (Jubalo®, Seelbach, Germany) using microcells high-precision cell quartz with 10-mm light path (Hellma Analytics, Müllheim, Germany). Fluorometric assays were recorded on a Safas Xenius® (Safas Monaco) using Corning® Costar 96-wells black plates with clear bottom. Preparative flash column chromatography was performed on a CombiFlash Companion using GraceResolv Silica cartridges. NMR spectra were recorded on a Bruker 600 MHz spectrometer (Evry University, France). Chemical shifts (expressed in ppm) of ^{1}H and ^{13}C NMR spectra were referenced to the solvent peaks δ (H) = 7.24 and δ (C) = 77.2 for $CDCl_3$.

Standard synthesis

Synthesis of *N*-methylbutyrylamide (4). Standard amide **4** was synthesized according to a similar procedure as described.[20] To a solution of butyric acid (**1**) (2.00 mmol, 176.6 mg) in dried dichloromethane (DCM) (7 mL) was added 4-dimethylaminopyridine (DMAP) (0.41 mmol, 0.2 equiv., 50.5 mg) followed by *N*-ethyl-*N*'-(3-dimethylaminopropyl) carbodiimide HCl (EDC.HCl) (2.24 mmol, 1.1 equiv., 462.4 mg), methylamine HCl (2.42 mmol, 1.2 equiv., 163.2 mg)

and trimethylamine (5.00 mmol, 2.5 equiv., 506 mg) at 0°C. The mixture was stirred under inert atmosphere at room temperature for 24 h and then quenched with 20 mL saturated ammonium chloride solution. The aqueous layer was extracted with DCM (3 x 15 mL). The combined organic layers were washed with brine (10 mL), dried over magnesium sulfate, filtered and concentrated under reduced pressure to dryness. Purification of the product by flash chromatography on silica gel (gradient mode: 50/50 ethyl acetate/petroleum ether to 100% ethyl acetate) afforded the desired amide as a yellow oil; 41% yield (83.4 mg). ^{1}H NMR ($CDCl_3$, 600 MHz): δ [ppm] = 2.81 (d, J = 5.1, 3H), 2.15 (t, J = 7.4, 2H), 1.66 (m, J = 7.4, 2H), 0.94 (t, J = 7.4, 3H); ^{13}C NMR ($CDCl_3$, 150 MHz): δ [ppm] = 173.7 (s), 38.6 (s), 26.2 (s), 19.2 (s) (Figures S8 and S9).

Synthesis of *S*-butyryl-*N*-acetylcysteamine. The same protocol as the one described for **4** was used, with *N*-acetylcysteamine (2.4 mmol, 1.2 equiv., 286.04 mg) in place of methylamine HCl. Purification of the product by flash chromatography on silica gel (gradient mode: 60/40 ethyl acetate/petroleum ether to 100% ethylacetate) afforded the desired product as a colorless oil; yield 60% (225 mg). ^{1}H NMR ($CDCl_3$, 600 MHz): δ [ppm] = 3.43 (q, J = 6.2, 2H), 3.03 (t, J = 6.4, 2H), 2.56 (t, J = 7.4, 2H), 1.97 (s, 3H), 1.70 (m, J = 7.5, 2H), 0.96 (t, J = 7.4, 3H); ^{13}C NMR ($CDCl_3$, 150 MHz): δ [ppm] = 200.1 (s), 170.3 (s), 45.9 (s), 39.8 (s), 28.4 (s), 23.2 (s), 19.2 (s), 13.5 (s) (Figures S10 and S11).

Enzyme expression and purification

All steps from primers purchase to cell lysate preparations were carried out as previously described.[21] The enzymes were purified by loading the cell-free extract onto a Ni-NTA column (QIAGEN) according to the manufacturer's instructions. The elution buffer was 50 mM phosphate (pH 7.5), 50 mM NaCl, 250 mM imidazole and 10% glycerol and the desalting buffer was 50 mM phosphate (pH 7.5), 50 mM NaCl and 10% glycerol. For the PPK2-III, purification by heat treatment has been used: cell-free extracts (50 mL) were heated at 70°C for 20 min. After centrifugation (12000 rpm, 4°C, 20 min), the supernatant containing the purified enzyme was taken. Large-scale purification (*Msed*CoAlig$_1$, *Gthe*CoAlig, *Dgeo*PPK2-III and *Ph*PPase) were conducted from a 500 mL culture by nickel affinity chromatography in tandem with gel filtration (Hi Load 16/60 Superdex 200pg 17-1069-01) for *Gthe*CoAlig and with desalting (HiPrep 26/10 17-5087-01) for *Msed*CoAlig$_1$, *Dgeo*PPK2-III and *Ph*PPase as described elsewhere.[22] The storage buffer was 50 mM phosphate pH 7.5, 50 mM NaCl, 10% glycerol and 1 mM DTT for *Msed*CoAlig$_1$ and 50 mM MOPS pH 8.0, 50 mM NaCl, 10% glycerol and 1 mM DTT for *Gthe*CoAlig, *Dgeo*PPK2-III and *Ph*PPase. Protein concentrations were determined by the Bradford method with bovine serum albumin as the standard.[23] The samples were analysed by SDS-PAGEs using the Invitrogen NuPage system (Figures S12-S14). The purified proteins were stored at -80°C.

UHPLC-MS/UV analysis

UHPLC-MS analyses were performed using a Kinetex® EVO-C18 (Phenomenex) column (100 x 2.1 mm; 1.7 µm). Conditions A: Mobile phase was ammonium formate pH 8.8 10 mM as solvent A and MeOH as solvent B. A linear gradient was applied (ratio A/B 70/30 to 50/50 in 10 min; then 50/50 to 20/80 in 1 min, 20/80 during 2 min and then 20/80 to 70/30 in 30 s); flow 0.3 mL.min^{-1}; temperature 25°C; injection volume 3 µL; MS detection. Conditions B: Mobile phase was H_2O + 0.1% formic acid as solvent A and MeCN as solvent B. A linear gradient was applied (ratio A/B 95/5 during 2 min, then 95/5 to 0/100 in 7 min, then 0/100 during 1 min, 0/100 to 95/5 in 1 min, 95/5 during 1 min); flow 0.3 mL.min^{-1}; temperature 25°C; injection volume 3 µL; MS detection. HPLC-UV analyses were performed using a Zic® pHilic (Merck) column (150 x 4.6 mm; 5 µm). Conditions C: Mobile phase was ammonium carbonate pH 9.9 10 mM as solvent A and MeCN as solvent B. A linear gradient was applied (ratio A/B 20/80 during 2 min, then 20/80 to 60/40 in 20 min; then 60/40 during 8 min and then 60/40 to 20/80 in 5 min); flow 0.5 mL.min^{-1}; temperature 25°C; injection volume 10 µL; UV detection at λ = 260 nm.

Analytical reaction monitoring

All the reactions were performed in a 100 µL final volume containing the specified buffer, carboxylic acid substrate, amine, specified cofactors, specified divalent cations, specified polyphosphate, and enzymes. The reactions were stirred at 400 rpm in a ThermoMixer® (Eppendorf) thermostated at the specified temperature. After the specified period of time (2, 7 or 24 h), an aliquot (30 µL) was quenched with 2 µL HCl 6 M and diluted with 100 µL H_2O and filtered on a 0.22 µm filter. The resulting mixtures were analysed by UHPLC-MS using conditions A for thioester intermediates monitoring, *ie* reactions with Nac or CoA (t_R (butyrylCoA) = 1.4 min, t_R (butyrylNac) = 4.1 min, t_R (*N*-methylbutyrylamide) = 1.2 min) and conditions B for amide monitoring (t_R (*N*-methylbutyrylamide) = 1.8 min, t_R (*N*-pentylbutyrylamide) = 7.1 min, t_R (*N*-cyclohexylbutyrylamide) = 6.4 min, t_R (*N*-phenethylbutyrylamide) = 7.4 min, t_R (*N*-isobutylbutyrylamide) = 3.5 min, t_R (*N*-ethylbutyrylamide) = 1.5 min, t_R (*N*-benzylbutyrylamide) = 4.6 min, t_R (butyrylglycine) = 1.7 min, t_R (butyrylalanine) = 2.4 min, t_R (*N*-methoxy-*N*-methylbutyrylamide) = 3.6 min). Negative control reactions in the absence of the enzyme or substrate were performed in parallel. All the results are the average of duplicated experiments. Conversions were deduced from calibration curves in duplicates obtained with commercial butyrylCoA (**3**), and with butyrylNac and *N*-methylbutyrylamide (**4**) synthesized according to the protocols described above. Calibration curve and UHPLC-MS chromatogram and MS spectra of reactions described in this paper are detailed in figures S15-S26.

AMP/ADP/ATP monitoring

AMP, ADP and ATP quantifications were monitored using conditions C: t_R (AMP) = 11.5 min, t_R (ADP) = 13.5 min, t_R (ATP) = 14.7 min. Corresponding HPLC-UV chromatograms are detailed in figures S27-S28.

Specific activities measurements

The appropriate amount of CoA ligase was added to a thermostated spectrophotometric cell preincubated for 2 min at 50°C containing 50 mM Tris buffer pH 8.0, 0.05 mM CoA, 0.25 mM ATP, 1 mM $MgCl_2$ and 3 mM substrate in a final volume of 100 µL. Tris buffer was used instead of MOPS buffer since this latter absorbs at 232 nm. The initial slope measured at 232 nm (absorbance of thioester bond), after addition of the purified CoA ligase, determined the specific activity of the enzyme according to Beer-Lambert's law and the molar absorptivity of butyrylNac determined in house (ε = 3.8.10^3 L.mol^{-1}.cm^{-1}) after subtraction of the slope obtained under the same conditions except without substrate and/or without enzyme. The same protocol was used for the temperature profile, except that the cell was preincubated at the specified temperature.

Fluorometric analysis

PPases activities were measured at 542 V of emission with a Sigma Aldrich Pyrophosphate Assay Kit (Catalog number: MAK169). See more details in SI (Table S3).

Lab-scale chemoenzymatic synthesis of *N*-methylbutyrylamide (4)

Into a 50 mL-erlenmeyer with a skirted stopper was poured 10 mL of a medium containing 50 mM MOPS buffer pH 8.5, 10 mM (1 mL from a solution of 100 mM) butyric acid (**1**), 100 mM (2 mL from a solution of 500 mM) methylamine, 0.5 mM (50 µL from a solution of 100 mM) ATP, 2 mM (0.2 mL from a solution of 100 mM) $PolyP_{25}$, 10 mM (1 mL from a solution of 100 mM) $MnCl_2$, 10 mM (1 mL from a solution of 100 mM) $MgCl_2$, 1 mg (116 µL from a solution of 8.6 mg/mL, 1.63 µM) *Gthe*CoAlig, 0.2 mg (408 µL from a solution of 0.49 mg/mL, 0.66 µM) *Dgeo*PPK2-III and 0.02 mg *Ph*PPase (168 µL from a solution of 0.12 mg/mL, 0.1 µM). The reaction mixture was stirred at 90 rpm in an Infors® incubation shaker at 60°C for 24 h and then quenched with 10 mL of a saturated solution of ammonium chloride. The aqueous layer was extracted with ethyl acetate (4 x 5 mL) and DCM (4 x 5 mL). The combined organic layers were washed with saturated sodium carbonate (10 mL), dried over magnesium sulfate, filtered and concentrated under reduced pressure to dryness to get a crude yellow oil (7.8 mg, crude yield = 77%). The TN was calculated as the moles of product divided by the moles of catalyst. NMR analyses were identical to those of the synthetic standard (*vide supra*, "Synthesis of *N*-methylbutyrylamide (4)").

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Keywords: amides • ATP regeneration • biocatalysis • cascade reaction • CoA ligase

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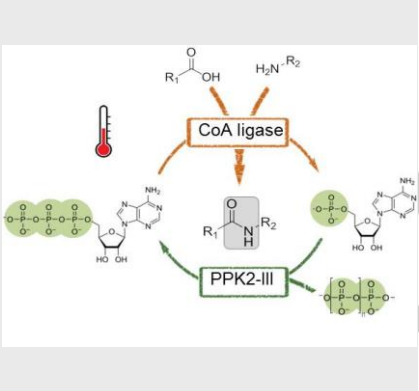

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