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Enzyme-Catalyzed Laurolactam Synthesis *via* Intramolecular Amide Bond Formation in Aqueous Solution

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Abstract: Lactam formation from ω-aminocarboxylic acids is thermodynamically unfavored in aqueous solution and therefore hard to achieve. In the present work ω-laurolactam hydrolases from *Acidovorax* sp. T31 and *Cupriavidus* sp. U124 were investigated regarding their potential to catalyze lactam formation. Both enzymes are known to hydrolyze laurolactam to 12-aminododecanoic acid. The ω-laurolactam hydrolase genes were expressed in *Escherichia coli* BL21 (DE3) and the catalytic activity of the respective proteins was investigated. As expected from thermodynamics, only laurolactam hydrolysis but not 12-aminododecanoic acid cyclization was observed in whole-cell biotransformations and cell extract assays. The utilization of 12-aminododecanoic acid methyl ester, as an activated form of 12-aminododecanoic acid, resulted in intramolecular amide bond formation with the product laurolactam. Maximum laurolactam formation rates of 13.5 and 14.3 $U g_{CDW}^{-1}$ and molar yields of 11.5% and 13.0% were achieved in biotransformations at pH 10 with recombinant *E. coli* harboring the ω-laurolactam hydrolase from *Cupriavidus* sp. U124 and *Acidovorax* sp. T31, respectively. Furthermore, it was shown that under the harsh reaction conditions applied, the utilization of whole-cell biocatalysts enables 17.2-fold higher laurolactam formation activity in comparison to free enzymes in solution. This study shows that hydrolase-catalyzed laurolactam synthesis can be achieved in aqueous solution by selection of an appropriate substrate and reaction pH.

Keywords: hydrolase; kinetically controlled amide synthesis; lactam formation; laurolactam; whole-cell biocatalysis

Introduction

Intermolecular amide bond formation can be performed in chemical or enzymatic reactions and is well established for the synthesis of medium size peptides.[1–4] In contrast, there are only few chemical or enzymatic approaches describing an intramolecular amide bond formation, e.g., for the synthesis of lactams. The formation of lactams *via* intramolecular ring closure reaction of the corresponding ω-aminocarboxylic acids is entropically unfavored and therefore difficult to achieve.[5]

Rico et al. reported the utilization of *N*-hexadecyl-2-chloropyridinium iodide (C_{16}PyCl,I) as an activating agent to facilitate the ring closure reaction of 12-aminododecanoic acid to laurolactam.[5] Major disadvantages are the use of toxic compounds, such as 1,2-dichloroethane or triethylamine, the application of C_{16}PyCl,I in stoichiometric amounts, and intermolecular dimerization or polymerization reactions. Another approach to synthesize C_6 to C_{13} ring lactams from corresponding ω-aminocarboxylic acids utilizes chemical solid-phase peptide formation.[6] However, the requirement for toxic solvents and protective groups makes the process laborious and environmentally unfriendly.

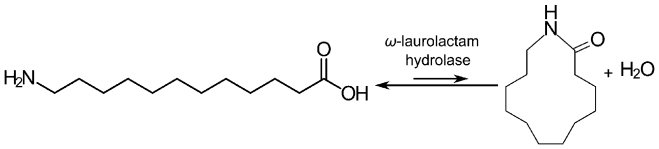
Enzyme-catalyzed intramolecular amide formation from ω-amino esters can be an alternative to chemical syntheses as described for γ- and δ-lactam synthesis using porcine liver esterase in aqueous solution.[7] Compared to chemical synthesis, the enzymatic reaction takes place under mild reaction conditions with reduced production of toxic waste.

Enzyme-catalyzed syntheses can either be carried out with free enzymes or with whole cells containing the enzymes. Both approaches are widely used for the

production of chemicals and pharmaceuticals.[2,3,8–10] The choice for one of these options depends on the reaction itself, enzyme stability, and costs of enzyme isolation and purification. Natural encapsulation inside cells protects the enzyme from inactivating agents or shear forces[2,3,11] and may increase enzyme stability, even when the cell is metabolically inactive.[12] However, diffusion of substrates over the cell membrane may limit mass transfer in whole-cell systems, which often results in reduced catalytic activity compared to free enzymes.[13]

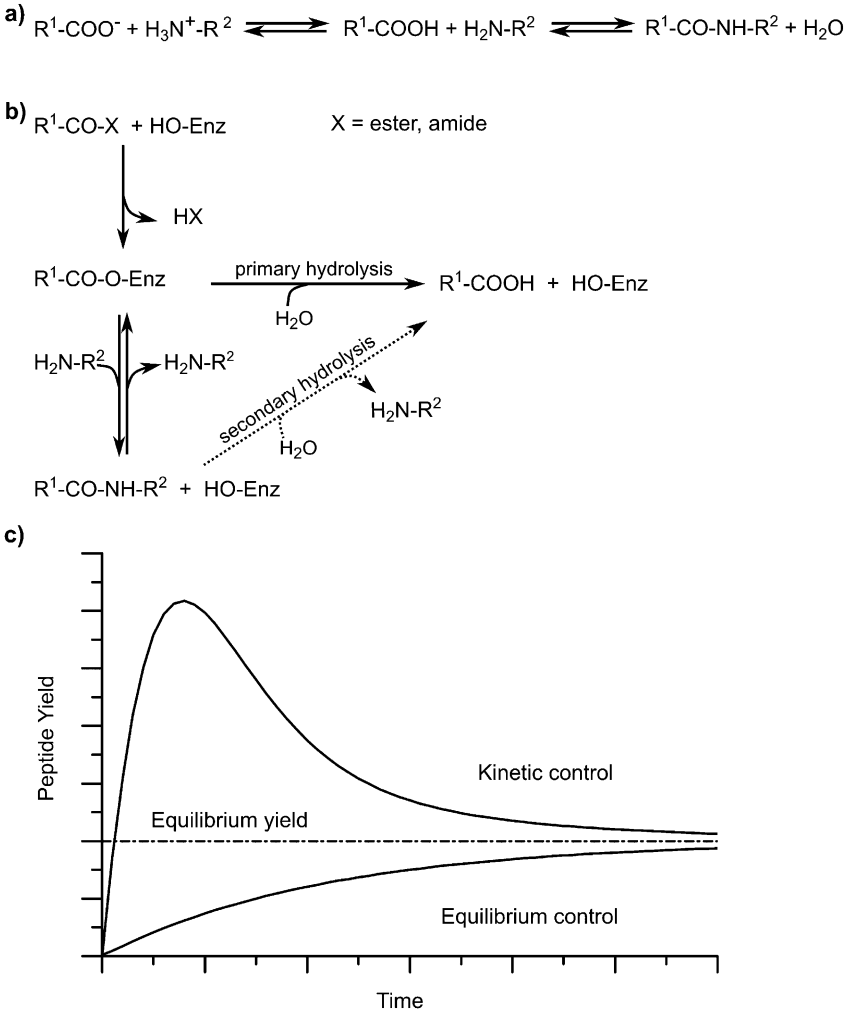
Recently, laurolactam hydrolysis activity has been reported in several soil bacteria, and ω-laurolactam hydrolases were identified as responsible enzymes.[14] In cell-free extracts of recombinant *E. coli* JM109 harboring the ω-laurolactam hydrolases from either *Acidovorax* sp. T31 or *Cupriavidus* sp. U124, 12-aminododecanoic acid formation rates of 2.77 and 1.24 U $mg_{protein}^{-1}$ were observed, respectively.[15]

In this study, these ω-laurolactam hydrolases were investigated regarding their potential to catalyze intramolecular amide bond formation and thus to convert 12-aminododecanoic acid to laurolactam serving as the model reaction (Scheme 1).



Scheme 1. Condensation reaction of 12-aminododecanoic acid to laurolactam catalyzed by an ω-laurolactam hydrolase.

The catalytic behavior of the ω-laurolactam hydrolases was evaluated following the equilibrium controlled and kinetically controlled reaction concepts, which originally have been developed for peptide synthesis[16,17] (Scheme 2). The pH optimum of the lactam formation reaction was investigated using whole cells and cell extracts.



Scheme 2. Equilibrium (**a**) and kinetically controlled (**b**) peptide synthesis and their time dependent yield (**c**). Adapted from Wegmann et al. and Heyland et al.[9,10]

Results and Discussion

Construction and Characterization of Recombinant *E. coli* Strains

In order to achieve and evaluate the expression of the ω-laurolactam hydrolase genes from *Acidovorax* sp. T31 and *Cupriavidus* sp. U124 in recombinant *E. coli* BL21 (DE3), the expression vectors pCom10_T31 and pCom10_U124 were constructed and biocatalytic activities for laurolactam hydrolysis were determined. Laurolactam hydrolysis was tested in biotransformations using whole cells or crude extracts.

Compared to *E. coli* BL21 (DE3) (pCom10_U124), *E. coli* BL21 (DE3) (pCom10_T31) showed about 1.4- and 1.2-fold higher 12-aminododecanoic acid formation rates *in vivo* and *in vitro*, respectively (Table 1).

After complete consumption of laurolactam, the yield of 12-aminododecanoic acid was greater than 99%. Assuming a whole cell protein content of 55% of the bacterial cell dry mass,[18] the observed *in vitro* activities of $177.7 \pm 3.7\ U\ g_{protein}^{-1}$ and $149.7 \pm 1.5\ U\ g_{protein}^{-1}$ correspond well with the experimentally determined *in vivo* activities (Table 1). This indicates that mass transfer of laurolactam over the cell membrane was not limiting. Wild-type *E. coli* BL21 (DE3) did not catalyze laurolactam hydrolysis (data not shown) confirming that laurolactam hydrolysis resulted from ω-laurolactam hydrolase activity and not from intrinsic enzyme activities.

Laurolactam Synthesis from 12-Aminododecanoic Acid

After the activity of ω-laurolactam hydrolases in recombinant *E. coli* BL21 (DE3) had been shown, 12-aminododecanoic acid cyclization to laurolactam was investigated. Laurolactam formation was not observed in biotransformations of 12-aminododecanoic acid using whole cells and cell extracts at pH 7.4.

Following the concept of equilibrium controlled peptide synthesis, amide bond formation can be promoted by increasing the amount of non-protonated amino acids in solution.[16,17,19] This can be achieved by increasing the pH of the reaction medium. However, a $pH > 8$ generally results in ω-laurolactam hydrolase deactivation.[14] To find a compromise between enzyme deactivation at higher pH and providing a sufficient amount of non-protonated amino species for the reaction, biotransformations were performed at pH 10. However, laurolactam formation was also not observed in whole-cell biotransformations at pH 10.

Amide bond formation also depends on the concentration of the uncharged carboxylic acid group of the substrates.[16] Since about 99% of the carboxylic acid groups of 12-aminododecanoic acid are present as anions at $pH \geq 7.4$ (Marvin Software, ChemAxon Ltd. Budapest, Hungary), the formation of the acyl-enzyme is inhibited and thus the ring closure reaction to the lactam is hindered.

Laurolactam Synthesis Following a Kinetically Controlled Reaction Concept

According to the kinetically controlled reaction concept (Scheme 2), activated substrates can be used to achieve protease-catalyzed peptide formation.[16,17] Activated substrates are ω-aminocarboxylic acids with a substituted carboxylic acid group such as, for example, amides or esters, which provide good leaving groups to form a covalent acyl-enzyme intermediate within serine or cysteine hydrolases. This intermediate is attacked by a nucleophile and the acyl group is transferred.[16,19] The investigated ω-laurolactam hydrolases from *Acidovorax* sp. T31 and *Cupriavidus* sp. U124 show 98% amino acid sequence homology to the serine hydrolase NylA from *Arthrobacter* sp. and contain the catalytic triad typical for serine hydrolases.[14,20] Thus, following the kinetically controlled reaction concept seemed to be a feasible approach for laurolactam synthesis with the ω-laurolactam hydrolases.

For this purpose, 12-aminododecanoic acid methyl ester was chosen as activated substrate. Whole-cell biotransformations were performed with *E. coli* BL21 (DE3) (pCom10_T31) and *E. coli* BL21 (DE3) (pCom10_U124) at pH 7.4. Formation of laurolactam was not detected. Only ester hydrolysis to 12-amino-

Table 1. 12-aminododecanoic acid formation activities and yields for laurolactam with recombinant *E. coli* BL21 (DE3).

	BL21 (DE3) (pCom10_T31)		BL21 (DE3) (pCom10_U124)	
	Initial rates[a]	Acid yield[b]	Initial rates[a]	Acid yield[b]
In vivo	$96.5 \pm 1.2\ U\ g_{CDW}^{-1}$	>99%	$68.3 \pm 1.0\ U\ g_{CDW}^{-1}$	>99%
In vitro[c]	$177.7 \pm 3.7\ U\ g_{protein}^{-1}$	>99%	$149.7 \pm 1.5\ U\ g_{protein}^{-1}$	>99%

[a] Initial rates are based on product formed within the initial 5 min of reaction.
[b] Conversion of 1.5 mM laurolactam to 12-aminododecanoic acid within 60 min (*in vivo*) and 30 min (*in vitro*).
[c] Specific activity is calculated based on whole-cell protein content.

dodecanoic acid was observed. The initial rates were determined to be $80.3 \pm 5.6\ U\ g_{CDW}^{-1}$ with *E. coli* BL21 (DE3) (pCom10_T31) and $84.2 \pm 1.9\ U\ g_{CDW}^{-1}$ with *E. coli* BL21 (DE3) (pCom10_U124). In control experiments with wild-type *E. coli* BL21 (DE3), ester hydrolysis occurred at initial rates of $1.7 \pm 1.1\ U\ g_{CDW}^{-1}$ (data not shown). Thus, the hydrolysis of 12-aminododecanoic acid methyl ester at pH 7.4 can primarily be attributed to ω-laurolactam hydrolase activities.

Since the kinetically controlled approach did not result in laurolactam formation at pH 7.4, the pH of the reaction medium was adjusted to pH 10 to increase the amount of non-protonated amine species to 38% (Marvin Software, ChemAxon Ltd., Budapest, Hungary). Biotransformations of 12-aminododecanoic acid methyl ester were performed with *E. coli* BL21 (DE3) (pCom10_T31) and *E. coli* BL21 (DE3) (pCom10_U124) for 2 h and indeed resulted in laurolactam formation (Figure 1) as confirmed by GC-MS analysis (Supporting Information).

The substrate was completely converted to 12-aminododecanoic acid and laurolactam within 60 min. Molar laurolactam yields of 13.0 and 11.5% were obtained after 30 min with *E. coli* BL21 (DE3) (pCom10_T31) and *E. coli* BL21 (DE3) (pCom10_U124), respectively, constituting a transient maximum followed by a decrease due to secondary hydrolysis as expected for kinetically controlled amide bond formation. Both strains showed similar initial laurolactam formation ($14.4 \pm 0.7\ U\ g_{CDW}^{-1}$ and $13.5 \pm 1.1\ U\ g_{CDW}^{-1}$, respectively) and ester hydrolysis activities ($63.2 \pm 2.3\ U\ g_{CDW}^{-1}$ and $54.6 \pm 1.8\ U\ g_{CDW}^{-1}$, respectively). The balance of substrate and product

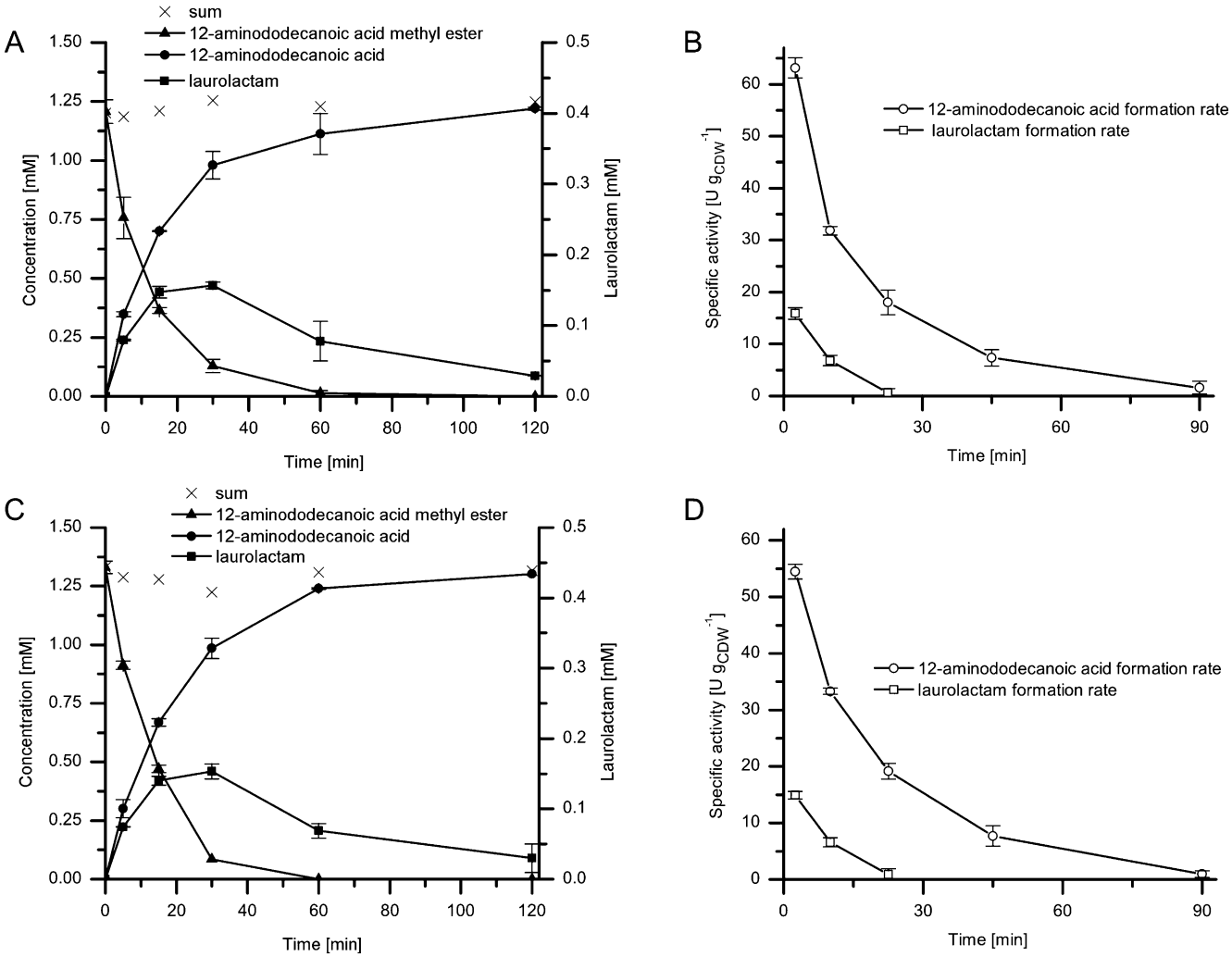


Figure 1. Substrate depletion and product formation patterns (**A** and **C**) and corresponding specific activities (**B** and **D**) observed during whole-cell biotransformations of 12-aminododecanoic acid methyl ester with *E. coli* BL21 (DE3) (pCom10_T31) (**A** and **B**) and *E. coli* BL21 (DE3) (pCom10_U124) (**C** and **D**) at pH 10. A cell concentration of $1.2\ g_{CDW}\ L^{-1}$ was used. The results shown were obtained from duplicate samples and were confirmed by repetition of the experiment.

concentrations was closed and no additional peaks were observed by GC and HPLC analysis, clearly indicating that no product degradation by activities of host intrinsic enzymes occurred and no other side products such as intermolecular amides were formed.

In contrast to the biotransformation of laurolactam at pH 7.4 (Table 1), the lactam was not completely hydrolyzed to 12-aminododecanoic acid. About 30 μM laurolactam remained after 2 h of biotransformation. A pH-driven shift of the thermodynamic equilibrium to an equilibrium concentration of around 30 μM laurolactam can be excluded as a reason for the lowered laurolactam degradation, as laurolactam formation from 12-aminododecanoic acid was not detected at pH 10. Hence, the reduced laurolactam hydrolysis rates are most likely due to a loss of enzymatic activity caused by the high pH applied.

At pH 10, wild-type *E. coli* BL21 (DE3) catalyzed ester hydrolysis at a rate of $7.2 \pm 0.9\ U\ g_{CDW}^{-1}$. Laurolactam formation was not detected. As abiotic control, 1.0 mM 12-aminododecanoic acid methyl ester was dissolved in the reaction buffer and shaken under biotransformation conditions. A spontaneous laurolactam formation was not detected. However, ester hydrolysis to 12-aminododecanoic acid occurred at a rate of $0.8\ \mu M\ min^{-1}$ (data not shown). Thus, laurolactam formation can be solely ascribed to ω-laurolactam hydrolase activity.

The pH value of the reaction medium obviously is a critical factor for enzyme-catalyzed laurolactam formation. Hence, the pH dependency of whole cell-catalyzed laurolactam formation was investigated. Up to pH 10, laurolactam formation rates increased in correlation to the pH (Figure 2). A further increase of the pH to 10.5 resulted in a severe drop of the specific activity to around $0.5 \pm 0.1\ U\ g_{CDW}^{-1}$. At pH 11, no laurolactam formation was observed. Between pH 8 and 9.5, maximum laurolactam yields of around 2–3% were observed after 5 min of biotransformation followed by a decrease in laurolactam concentrations due to secondary hydrolysis. At pH 10, the non-protonated substrate acted as an effective nucleophile in the formation of the lactam, whereas pH values higher than 10 drastically reduced the laurolactam yield, most likely due to enzyme inactivation. Consequently, a pH value of 10 was found to be optimal for enzyme catalyzed laurolactam formation from 12-aminododecanoic acid using whole cells.

Whole-Cell Biocatalysis Enhances Enzyme Performance

To compare the catalytic performance of whole cells and free enzymes, 12-aminododecanoic acid methyl ester biotransformations were performed with cell extracts of *E. coli* BL21 (DE3) (pCom10_T31) at pH 10 (Figure 3).

Again, the substrate was completely converted to 12-aminododecanoic acid and laurolactam within 60 min. Laurolactam was formed to a lower maximum concentration of 65 μM (maximum yield of 6.3%) within 30 min and was only slightly degraded to 56 μM within 3 h. A maximum laurolactam formation rate of $1.5 \pm 1.0\ U\ g_{protein}^{-1}$ was obtained corresponding to approximately $0.83\ U\ g_{CDW}^{-1}$. Ester hydrolysis to 12-aminododecanoic acid was observed at a maximum rate of $25.0 \pm 1.4\ U\ g_{protein}^{-1}$ (*ca.* $13.8\ U\ g_{CDW}^{-1}$).

Whole-cell biotransformations and crude extract assays show clearly different bioconversion character-

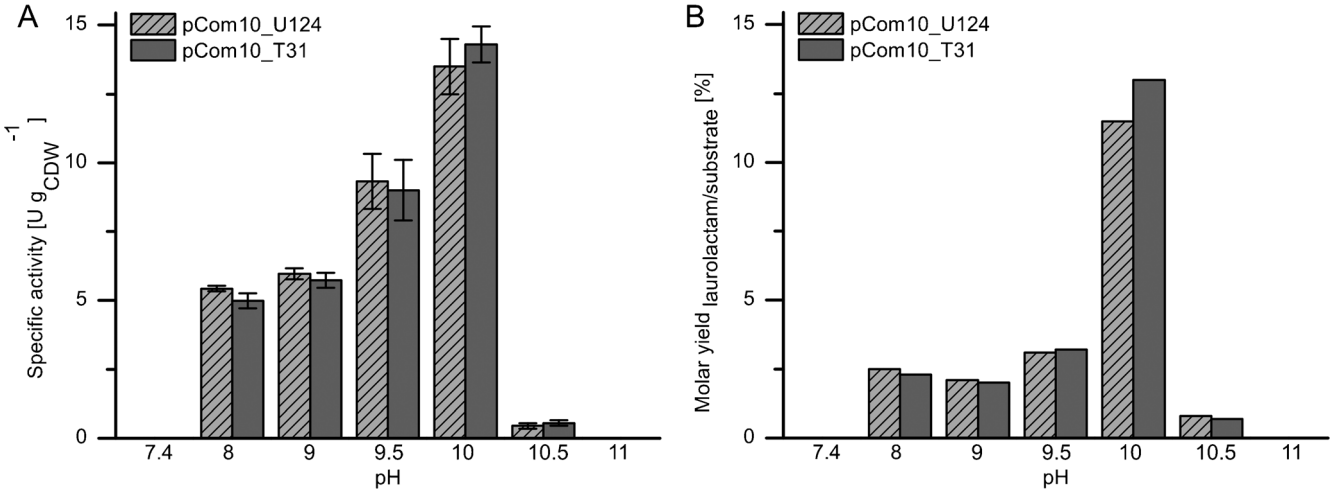


Figure 2. Specific laurolactam formation rates (**A**) and maximally achieved laurolactam yields (**B**) during whole-cell biotransformations of 1.5 mM 12-aminododecanoic acid methyl ester with *E. coli* BL21 (DE3) (pCom10_U124) and *E. coli* BL21 (DE3) (pCom10_T31). Maximal yields were obtained after 5 min of biotransformation at $8 \leq pH \leq 9.5$ and after 30 min of biotransformation at pH 10 and 10.5.

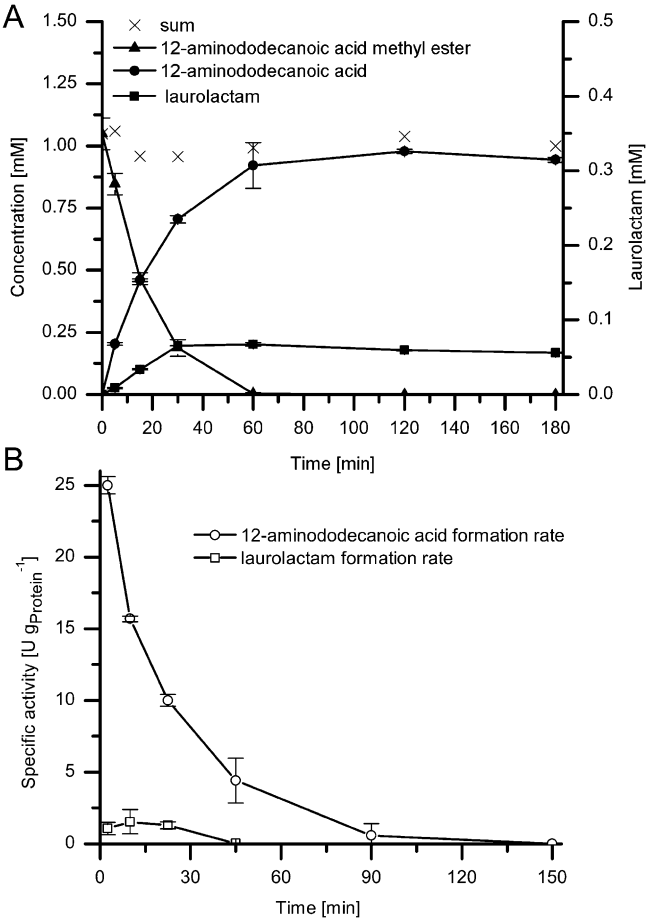


Figure 3. Substrate depletion and product formation patterns (**A**) and corresponding specific activities (**B**) observed during biotransformations of 12-aminododecanoic acid methyl ester at pH 10 with crude extract of *E. coli* BL21 (DE3) (pCom10_T31). A protein concentration of 1.63 g L^{-1} was used. The results shown were obtained from duplicate samples and were confirmed by repetition of the experiment.

istics at pH 10. In contrast to the distinct laurolactam concentration maximum observed in whole-cell biotransformations, the lactam was only scarcely degraded when cell extracts were applied. Maximally achieved laurolactam yields were 2.1-fold lower. Furthermore, maximum laurolactam and 12-aminododecanoic acid formation rates were lowered 17.2- and 4.6-fold, respectively. The different extent to which these rates were reduced may be explained by the fact that next to the ω-laurolactam hydrolase, intrinsic enzymes also catalyzed ester hydrolysis to 12-aminododecanoic acid. This becomes obvious from the control experiments with wild-type cells described above.

The results indicate that the ω-laurolactam hydrolase was deactivated at high pH. Hence, biotransformations were performed at pH values ranging from 7.4 to 11 to determine the pH dependency for laurolactam formation using cell extracts. Laurolactam formation rates obtained with crude extracts are compared to respective rates obtained with whole cells by translating the latter into corresponding *in vitro* values assuming a whole-cell protein content of 55% (Figure 4).

Using cell extracts, laurolactam formation was observed in biotransformations at pH values between 7.4 and 10. Maximum specific activities of 13.9 ± 0.64 U $g_{protein}^{-1}$ were observed at pH 8 and decreased with increasing pH, most likely due to ω-laurolactam hydrolase deactivation. At pH $\geq$ 10.5, no laurolactam was found. The enzyme appeared to be completely inactivated by the high pH applied. This is in accordance with results obtained by Asano and co-workers.[14] Similar to whole-cell biotransformations, the maximum laurolactam yield (6.3% after 30 min) was found at pH 10. At pH values between 8 and 9.5, lower laurolactam yields were obtained due to ester and lactam hydrolysis at high rates.

Enzyme-catalyzed laurolactam formation depends on several parameters: (i) the primary and secondary hydrolysis rate (Scheme 2), (ii) the nucleophilic attack of the non-protonated substrate, and (iii) enzyme deactivation at high pH values. Using whole cells, the hydrolysis rates (sum of primary and secondary hydrolysis) increased from 84.2 ± 1.9 U g_{CDW}^{-1} to 221.3 ± 3.1 U g_{CDW}^{-1} when increasing the pH from 7.4 to 9.5. At pH 10, the hydrolysis rates were drastically reduced to a minimum of 63 ± 2.3 U g_{CDW}^{-1} with the non-protonated substrate acting as an effective nucleophile. Thereby, the activity ratio of laurolactam synthesis to overall hydrolysis increased to a maximum of 26.4% (Figure 5). Such a high ratio was not found using crude extracts, since significant enzyme deactivation occurred at pH > 8 and primary and secondary hydrolysis took place at high rates (310.9 ± 9.7 U $g_{protein}^{-1}$) at pH 8.

In whole cells, the ω-laurolactam hydrolase seems to be protected from inactivation at least for a certain period of time. At pH $\leq$ 10, the bacterial pH regulation system might have adjusted the intracellular pH to a lower level and thus preserved enzymatic activity. For example, sodium proton antiporters (NhaA) or multidrug resistance transporters (MdfA) are involved in pH homeostasis in *E. coli*.[21,22] However, the application of an external pH higher than pH 9 also raises the intracellular pH above pH 8,[22] which generally results in deactivation of the ω-laurolactam hydrolase.[14] Furthermore, enzyme inactivation might have been counteracted by host intrinsic chaperones, which are able to refold denatured enzymes to their active conformation.[23] However, the pH regulation system of *E. coli* is compromised at pH > 9 and cell death occurs.[22,24] Small et al. investigated pH resistance of *E. coli* by determining the number of colony forming units after incubation at pH 10.2. It was shown that 15% of the cells survived for 2 h at pH 10.2.[25] These observations and the results ob-

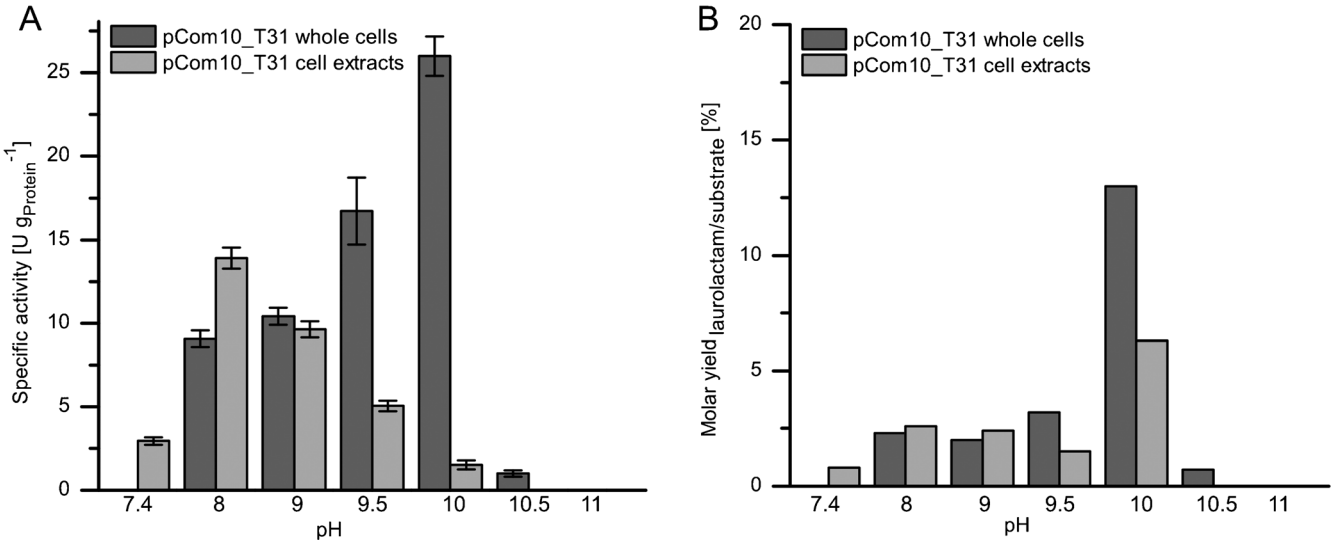


Figure 4. Specific laurolactam formation rates (**A**) and molar laurolactam yields (**B**) observed during biotransformations of 1.5 mM 12-aminododecanoic acid methyl ester with whole cells of *E. coli* BL21 (DE3) (pCom10_T31) and respective cell extracts. The rates obtained from whole-cell biotransformations were translated into their corresponding *in vitro* values, assuming a whole-cell protein content of 55%. Maximal yields were obtained after 5 min of biotransformation at $7.4 \leq pH \leq 9.5$ and after 30 min of biotransformation at pH 10 and 10.5.

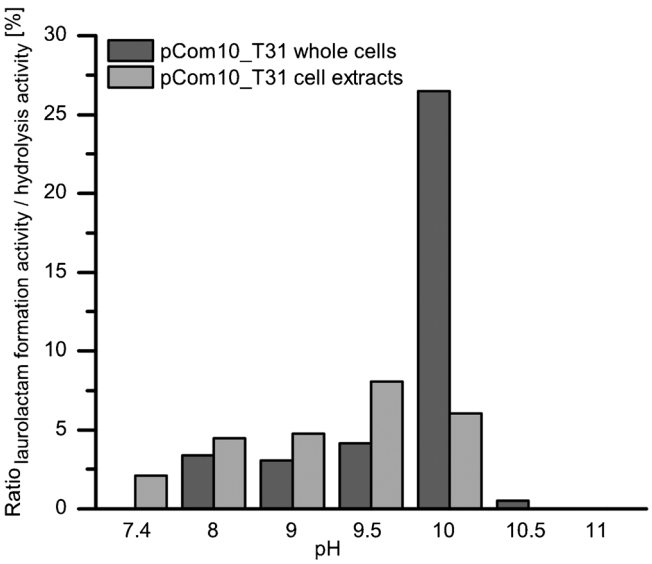


Figure 5. Ratios of initial activities for laurolactam formation and overall hydrolysis in biotransformations of 1.5 mM 12-aminododecanoic acid methyl ester with whole cells of *E. coli* BL21 (DE3) (pCom10_T31) and respective cell extracts in dependence of pH.

tained in this study suggest that the ω-laurolactam hydrolase was protected from rapid inactivation during whole-cell biotransformations of 12-aminododecanoic acid methyl ester at $pH \leq 10$, resulting in higher product formation rates as compared to rates observed with cell extracts. However, cell viability is expected to decrease over time resulting in an intracellular alkalization and thus in ω-laurolactam hydrolase inactivation. This is supported by the reduced laurolactam hydrolysis rates after 60 min observed during whole-cell biotransformations at pH 10 (Figure 1).

Future work will focus on the application of a two-liquid phase system, which represents a possible strategy to enhance the product yield[26] while operating at lower pH. By addition of a suitable organic phase, enabling the efficient *in situ* extraction of laurolactam, but not of 12-aminododecanoic acid, the conversion of the respective methyl ester may be directed towards laurolactam formation at the same time preventing laurolactam hydrolysis.

Conclusions

ω-Laurolactam hydrolases were tested for laurolactam formation from 12-aminododecanoic acid and 12-aminododecanoic acid methyl ester in aqueous solution. Laurolactam formation from 12-aminododecanoic acid could not be observed. However, applying the concept of kinetically controlled peptide synthesis to intramolecular amide bond formation, laurolactam formation from 12-aminododecanoic acid methyl ester was achieved. A maximum yield for laurolactam of 13.0% was obtained during whole-cell biotransformations using *E. coli* BL21 (DE3) (pCom10_T31) and the application of whole cells resulted in 17.2-fold higher laurolactam formation rates in comparison to free enzymes at pH 10. The ω-laurolactam hydrolase seemed to be protected in whole cells, whereas the free enzyme was deactivated. Thus, kinetically controlled amide bond formation proved to be a promising approach for lactam synthesis using recombinant cells.

Experimental Section

Strains, Plasmids and Growth Conditions

Laurolactam was purchased from TCI Europe (Zwijndrecht, Belgium) with a purity of >99%. 12-Aminododecanoic acid methyl ester with a purity ≥99% and 12-aminododecanoic acid with a purity of 96% were obtained from Evonik Degussa GmbH (Marl, Germany).

Strains and plasmids used in this work are listed in Table 2. *E. coli* DH5α was used for cloning purposes and *E. coli* BL21 (DE3) for recombinant gene expression and biocatalytic studies.

To obtain recombinant strains, plasmid DNA was introduced into the strains *via* electroporation (2.5 kV, EquiBio Easyjet Prima, Ashford, UK) and transformants were selected *via* their antibiotic resistance.

E. coli cells were grown either in lysogeny broth (LB)[30] or M9* minimal medium[30,31] containing 9 g KH_2PO_4, 25.5 g $Na_2HPO_4 \cdot 2\ H_2O$, 1 g NH_4Cl, 0.5 g NaCl, 0.49 g $MgSO_4 \cdot 7H_2O$, 5 g glucose, and 1 mL US^{Fe} trace element solution[32] per liter. Where appropriate, 50 mg L^{-1} kanamycin or 100 mg L^{-1} ampicillin were added. Solid media contained 1.5% (w/v) agar. Cultivation temperature was 30 or 37 °C as indicated. Liquid cultures were incubated in tubes or in baffled Erlenmeyer flasks in horizontal shakers at 200 rpm. Stock cultures were prepared by addition of 200 μL 50% (v/v) glycerol to 800 μL over-night grown LB cultures and stored at −80 °C.

Construction of pCom10_T31 and pCom10_U124

Restriction enzymes, Fast AP™ Thermosensitive Alkaline Phosphatase, and T4 Ligase were purchased from Fermentas (St. Leon-Rot, Germany) and used according to the supplier's protocols. Plasmid DNA was isolated with a peqGOLD Miniprep Kit I (PEQLAB Biotechnology GmbH, Erlangen, Germany) according to the supplier's recommendations. Genes encoding the ω-laurolactam hydrolases of *Acidovorax* sp. T31 (NCBI gene bank: AB444713) and *Cupriavidus* sp. U124 (NCBI gene bank: AB444714) were designed as follows: The gene sequence was not modified except for the start codon GTG, which was changed to ATG. The triplet CAT was introduced upstream of the gene sequence to form a *NdeI* restriction site together with the start codon. An additional multiple cloning site[29] containing a *Sal*I site was introduced downstream of the stop codon. The genes were synthesized by ATG:biosynthetics (Merzhausen, Germany) and delivered in the plasmids pBSK_T31 and pBSK_U124. The plasmids pBSK_T31, pBSK_U124, and pCom10 were digested with *Nde*I and *Sal*I. The pCom10 vector (7649 bp) and the pBSK-inserts (1500 bp) were isolated from agarose gels[30] and purified with a peqGOLD Gel Extraction Kit (PEQLAB Biotechnology GmbH, Erlangen, Germany). After dephosphorylation of pCom10 and ligation with the isolated pBSK_U124 or pBSK_T31 inserts, the DNA was transformed into *E. coli* DH5α *via* electroporation. Successful cloning yielding pCom10_T31 and pCom10_U124 was verified by restriction and sequence analysis of the insert region.

Whole-Cell Biotransformation

Whole-cell biotransformations with resting, i.e., non-growing but metabolically active, cells were performed to determine ω-laurolactam hydrolase activity. The specific activities are given in units per gram cell dry weight ($U\,g_{CDW}^{-1}$), whereby 1 U describes the activity forming 1 μmol product per minute. Cell concentrations were measured with a Libra S11 spectral photometer (Biochrom Ltd, Cambrigde, UK). A 5 mL LB preculture was inoculated with a single colony and incubated at 37 °C for 8 h. Fifty milliliters M9* medium were inoculated with 500 μL LB pre-culture and incubated overnight at 30 °C. Two-hundred milliliters M9* were inoculated with the M9* pre-culture to an optical density at 450 nm (OD_{450}) of 0.2 (1 $OD_{450} = 0.166\ g_{CDW}\,L^{-1}$[33]). The culture was incubated at 30 °C. At an OD_{450} of 0.5, induction with 0.025% (vol/vol) dicyclopropyl ketone was carried out and the cultivation was continued for 5 h. Cells were harvested by centrifugation (15 min, 4 °C, 4,595 × *g*; Heraeus Multifuge 1 S-R, Oberhausen, Germany) and resuspended either in 50 mM potassium phosphate buffer (pH 7.4) or 100 mM sodium carbonate buffer (pH 8, 9, 10, 10.5, 11) containing 1% glucose to a cell concentration of 1 $g_{CDW}\,L^{-1}$. Glucose was added to enable metabolism activity, e.g., for pH homeostasis. Aliquots of 1 mL were filled in Pyrex tubes and shaken at 30 °C and 350 rpm. After 5 min of adaptation, the reaction was initiated by the addition of either 1.5 mM laurolactam (from a 100 mM stock solution in ethanol) or 1.5 mM 12-aminododecanoic acid methyl ester (from a 60 mM stock solution in ethanol) or 0.75 mM 12-aminododecanoic acid (from a 50 mM stock solution composed of 50% (v/v) 0.1 N HCl in acetone). The reaction was stopped by addition of 1 mL ice-cold diethyl ether containing 0.2 mM dodecane as internal standard for gas chromatogra-

Table 2. *E. coli* strains and plasmids used in this study.

E. coli strain/ Plasmid	Characterization	References
BL21 (DE3)	F⁻, *ompT*, *hsdSB* ($r_B^-\ m_B^-$), λ(DE3 [*lacI lac*UV5 T7 gene *1 Sam7* Δ*nin5*])	[27]
DH5α	F⁻, *endA1*, *gln*V44, *thi-1*, *recA1*, *relA1*, *gyrA96*, *deoR*, *supE44*, Φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)-U169, *hsdR17* ($r_K^-\ m_K^+$), λ⁻	[28]
pBSK_T31	P15A *ori,* rep, T7 promoter, Amp^r, ω-laurolactam hydrolase gene from *Acidovorax* sp. T31	this study
pBSK_U124	P15A *ori,* rep, T7 promoter, Amp^r, ω-laurolactam hydrolase gene from *Cupriavidus* sp. U124	this study
pCom10	broad-host-ranged expression vector, *alk* promoter, Km^r	[29]
pCom10_T31	pCom10 with ω-laurolactam hydrolase gene from *Acidovorax* sp. T31	this study
pCom10_U124	pCom10 with ω-laurolactam hydrolase gene from *Cupriavidus* sp. U124	this study

phy (GC) or 0.5 mL acetonitrile for reversed phase high performance liquid chromatography (RP-HPLC) analysis.

Enzyme Activity Assay

Cells were grown, induced and harvested as described for whole-cell biotransformations. After harvesting, the cells from a 200 mL culture were resuspended in 5 mL 100 mM sodium carbonate buffer (pH 10) and disrupted by using a French press (three passages at 800 psi; SLM-Aminco, Rochester, NY, USA). Cell debris and non-lysed cells were removed by centrifugation (17,000 × *g*, 20 min, 4 °C; Fresco centrifuge, Heraeus, Oberhausen, Germany). The clarified cell extract was diluted ten-times with 100 mM potassium phosphate buffer (pH 7.4) or 100 mM sodium carbonate buffer (pH 8, 9, 9.5, 10, 10.5, 11) containing 1% glucose and used for activity assays. Aliquots of 1 mL of the diluted cell extracts were filled in Pyrex tubes and shaken at 30 °C and 400 rpm. After 5 min of adaption, substrate was added to a final concentration of 1.0 mM 12-aminododecanoic acid methyl ester (from a 40 mM stock solution in ethanol) or 1.5 mM laurolactam (from a 60 mM stock solution in ethanol). The reaction was stopped by addition of 1 mL ice-cold diethyl ether containing 0.2 mM dodecane as internal standard for GC analysis or with 0.5 mL acetonitrile or RP-HPLC analysis. Protein concentrations were determined using a commercially available Quick Start Bradford™ Protein Assay solution (Biorad, Munich, Germany) according to the supplier's protocol. Bovine serum albumin was used as standard protein.

Analysis of Metabolites

Laurolactam was analyzed *via* GC or GC-MS. For this purpose, a saturating amount of NaCl was added to the diethyl ether containing sample and it was vortexed for 1 min to extract laurolactam into the ether phase. After centrifugation (15 min, 4 °C, 4,595 × *g*), the organic phase was transferred into an Eppendorf cup and dried over anhydrous Na_2SO_4. The sample was centrifuged (2 min, 4 °C, 17,000 × *g*) and the ether phase was transferred into GC vials for analysis.

A Trace GC Ultra™ gas chromatograph (Thermo Fisher Scientific Inc., Waltham, MA, USA) equipped with a 30 m VF-5 ms FactorFour capillary column (5%-diphenyl and 95% dimethyl polysilphenylene-siloxane; inner diameter 0.25 mm; Varian, Middelburg, The Netherlands) and a flame ionization detector was used. A splitless sample injection was used. The initial temperature was set to 80 °C and was increased at a rate of 15 °C min^{-1} to 280 °C and then to 300 °C at 100 °C min^{-1}, which was kept for 2.5 min.

For GC-MS analysis, a CP-3800 gas chromatograph linked to a 1200 quadropole mass spectrometer (Varian Inc., Palo Alto, USA) and equipped with the same column as used for GC analysis. The sim mode was used to scan the specific mass fragments of *m/z* 30, *m/z* 41, *m/z* 55, *m/z* 86, *m/z* 98, *m/z* 100, *m/z* 112, *m/z* 126, *m/z* 140, *m/z* 154, *m/z* 168, and *m/z* 197. A splitless sample injection was used. The initial temperature was 80 °C. A temperature gradient of 15 °C min^{-1} was set until 160 °C were reached. Then, the temperature was raised to 250 °C at 10 °C min^{-1}, to 300 °C at 100 °C min^{-1}, and kept for 2.5 min.

The quantification of 12-aminododecanoic acid and 12-aminododecanoic acid methyl ester was performed *via* RP-HPLC analysis. The acetonitrile containing sample was vortexed for 1 min and centrifuged (15 min, 4 °C, 17,000 × *g*). The clear supernatant was analyzed. A 20 µL sample was injected onto a Luna C8(2) column (4.6 × 150 mm, 5 µm, 100 Å; Phenomenex®, Aschaffenburg, Germany) on a LaChrome Elite® HPLC system (VWR-Hitachi, Darmstadt, Germany), which was linked to a Corona charged aerosol detector (Dionex Softronic GmbH, Germering, Germany). The column temperature was set to 40 °C. A flow rate of 0.8 mL min^{-1} was applied. The mobile phases consisted of water containing 0.4% trifluoroacetic acid (TFA) (A), methanol (HPLC-grade) containing 0.2% TFA (B) and acteonitrile (HPLC-grade) (C). The following profile was applied: 0–2 min 45% A and 55% C, 2–24 min linear gradient to 25% A, and 75% C, 24–29 min linear gradient to 2% A, 30% B, and 68% C, 29–30 min linear gradient to 2% A, and 98% C, and 30–33 min linear gradient to 45% A and 55% C, which was kept for 2 min.

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