Importance of *N*-terminal proline for the promiscuous activity of 4-oxalocrotonate tautomerase (4-OT)

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Abstract: Michael addition of aldehydes to nitro-olefins is an effective method to obtain useful chiral *γ*-nitroaldehydes. *γ*-Nitroaldehydes are precursors for chiral *γ*-aminobutyric acid analogues, which have numerous pharmacological activities and are used for the treatment of neurological disorders. A whole-cell system based on recombinantly expressed 4-oxalocrotonate tautomerase (4-OT) was developed and shown to be an effective biocatalyst for the Michael addition of branched aldehydes to *β*-nitrostyrenes. The aim of this study was to investigate the influence of the substitution of the *N*-terminal proline with lysine and arginine, both containing a reactive *ε*-amino group, on the Michael addition catalyzed by 4-OT. First, the effects of these mutations were examined by *in silico* analysis, followed by the generation of three terminal lysine mutants. The generated mutants, 4-OT_K, 4-OT_PK and 4-OT_KK were tested for their ability to utilise *β*-nitrostyrene (**1**), (*E*)-1-nitro-2-(2-thienyl)ethene (**2**) and *trans*-*p*-chloro-*β*-nitrostyrene (**3**) as Michael acceptors with isobutanal (2-methylpropanal) as the donor. For comparison, the lithium salt of lysine was used in the same organocatalytic reactions. In general, the introduction of lysine had a negative effect on Michael additions based on overall product yields. However, additional lysine residues at the *N*-terminus of the protein resulted in structural changes that enhanced the activity towards **2** and **3.** Therefore, the N-terminal proline is important for 4-OT-catalysed Michael-additions, but it is not essential.

Keywords: 4-oxalocrotonate tautomerase, biocatalysis, organocatalysis, lysine, mutagenesis.

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INTRODUCTION

The enzyme 4-oxalocrotonate tautomerase (4-OT) is member of tautomerase superfamily with a unique catalytic *N*-terminal proline.[1,2] It is encoded by the xylH gene and is a part of an aromatic hydrocarbon degradation pathway in *Pseudomonas putida* mt-2. It catalyzes the conversion of 2-hydroxy-2,4-hexadienedioate to 2-oxo-3-hexenedioate.[3] In addition to its natural activity, 4-OT, when utilised as a free enzyme catalyses the isomerisation of *cis*-nitrostyrene to *trans*-nitrostyrene,[4] aldol condensations,[5] dehydrations, and Michael-type additions of acetaldehyde to *β*-nitrostyrene.[6] Michael-type reactions are widely utilised reactions for the formation of C–C bonds in general, and nitrostyrenes are highly active Michael acceptors that are often used in these type of reactions.[7–9] Michael-type addition of aldehydes to a variety of nitro-olefins is an effective method to obtain useful chiral *γ*-nitroaldehydes.[6,10–12] *γ*-Nitroaldehydes are precursors for chiral *γ*-aminobutyric acid analogues,[13] which have numerous pharmacological activities including antidepressant, anticonvulsant, anxiolytic, anti--emetic and other activities.[13,14]

Based on D_2O exchange and X-ray crystallography studies, it was revealed that the promiscuous activity of 4-OT relies on the deprotonation of acetaldehyde by the *N*-terminal proline and by the formation of enamine species between them.[15] Therefore, it is reasonable to propose that the reaction involving isobutanal proceeds *via* the same mechanism (Scheme 1A). Proline is unique because it is the only amino acid where the side chain is connected to the *α*-amino group, forming a five-membered nitrogen-containing ring, which makes proline an imino acid (Scheme 1B). It is aliphatic and hydrophobic, and has preference for a turn structure and can usually be found on the protein surface. The proline side-chain is non-reactive and due to the restricted dihedral angles, it can adopt in the polypeptide chain, it is rarely involved in the active site of an enzyme.[16] Proline
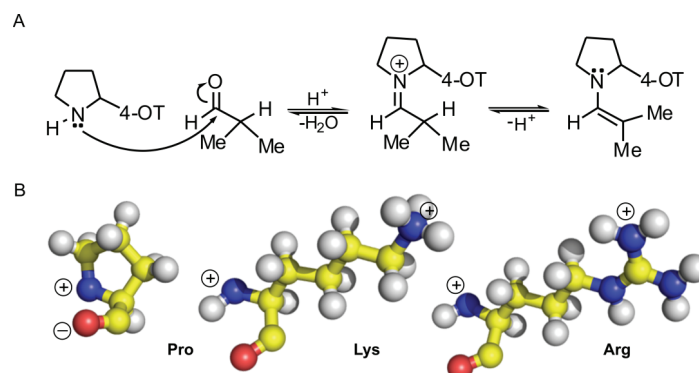
Scheme 1. A) Formation of the enamine intermediate in a Michael-type reaction and B) 3D structures and charges at physiological pH of the amino acids of interest (proline, lysine and arginine).

can sometimes be substituted with other small amino acids, although its unique properties limit the possible substitutions. Lysine and arginine are positively charged polar amino acids (Scheme 1B). They frequently play an important role in protein structure and are involved in salt-bridges, where they pair with a negatively charged amino acid to create a stabilizing form.[16] They are quite frequent in the active site of enzymes, because the positively charged ε-amino group on their side-chain is sometimes involved in electrostatic interactions.

A whole-cell system based on recombinantly expressed 4-OT has been developed in our laboratory and shown to be an effective biocatalyst for the asymmetric Michael addition of acetaldehyde and different branched aldehydes to β-nitrostyrenes.[11,17] Two mutants, one with an additional *N*-terminal proline (4-OT_P), as well as a variant with two substituted amino acids with proline, namely Ala3Pro and Gln4Pro (4-OT_2P), were generated and their activities assesed.[17] This study focuses on the effect of the substitution of the Pro1 of 4-OT (terminal proline (Pro1)) with basic amino acids containing ε-amino groups, namely lysine and arginine, through *in silico* analysis followed by generation of three specific 4-OT variants containing lysine. 4-OT variants were assessed for the ability to utilise β-nitrostyrene (**1**), (*E*)-1-nitro-2-(2-thienyl)ethene (**2**) and *trans-p*-chloro-β-nitrostyrene (**3**) as Michael acceptors with isobutanal as the Michael donor (Scheme 2). Additionally, an organocatalyst based on the lithium salt of lysine was prepared and utilized in the same reactions for an adequate comparison.
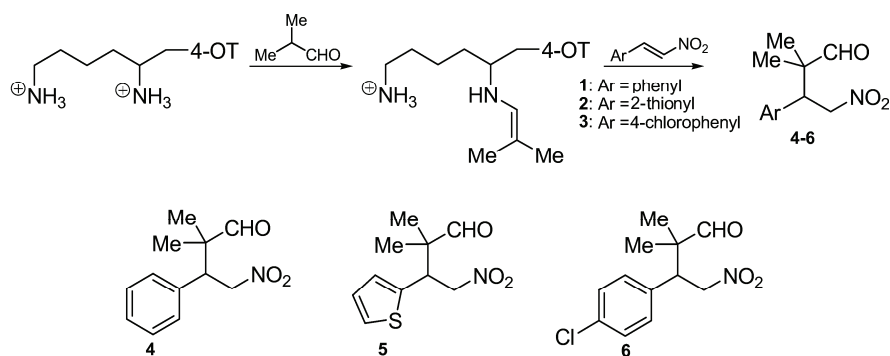
Scheme 2. Michael-type addition of isobutanal to substrate(s) **1–3** catalysed by whole-cell *Escherichia coli* BL21 (DE3) expressing 4-oxalocrotonate tautomerase, obtaining respective products **4–6**.

EXPERIMENTAL

Reagents

β-Nitrostyrene, (*E*)-1-nitro-2-(2-thienyl)ethane, *trans-p*-chloro-β-nitrostyrene, isobutanal, and all other chemicals were of analytical grade and purchased from Sigma–Aldrich (Munich, Germany). Ethyl acetate, ethanol and other solvents were of HPLC reagent grade and pur-

chased from Fisher Scientific (Hampton, NH, USA). Petrol ether (b.p. 50–70 °C) was purchased from Reahem, Novi Sad, Serbia.

The QuikChange® site-directed mutagenesis kit was purchased from Stratagene (La Jolla, CA, USA). The QIA-prep spin plasmid mini-prep kit was purchased from QIAGEN (Hilden, Germany). The BigDye® Terminator v1.1 Cycle Sequencing Kit was purchased from Applied Biosystems and primers from Invitrogen, both part of ThermoFisher Scientific (Foster City, CA, USA).

Ampicillin, isopropyl-β-D-1-thiogalactopyranoside (IPTG), and other salts and reagents were purchased from Sigma–Aldrich (Munich, Germany). Glucose, tryptone, yeast extract, casamino acids and other media components were purchased either from Oxoid (Cambridge, UK) or Becton Dickinson (Sparks, MD, USA).

4-OT molecular modelling

Mutated positions in the 4-oxalocrotonate tautomerase (4-OT) protein were mapped onto the available crystal structure (pdb code 1BJP) and the hexamer was formed according to the biologically active form of 4-OT. The protein structure was refined using the energy minimization script in CNS. Docking of substrate was performed in AutoDock Tools and Vina, figures were prepared in PyMol (The PyMOL Molecular Graphics System, Version 1.4.1., Schrodinger, LLC).

Mutagenesis

The recombinant *Escherichia coli* BL21(DE3) strain expressing 4-oxalocrotonate tautomerase was previously constructed and maintained in the laboratory.[17] Mutants were generated using the QuikChange® site-directed mutagenesis kit according to manufacturer's instructions using appropriate primers (Table I) for the introduction of single mutations. New plasmids were transformed into *E. coli* BL21(DE3). The recombinant plasmids were isolated and construct DNAs were verified by sequencing (Table I) using Applied Biosystems 3130 genetic analyzer (ThermoFisher Scientific, Foster City, CA, USA). The BLASTN program (NCBI, http://www.ncbi.nlm.nih.gov[18]) was used for sequence similarity searches.

TABLE I. Oligonucleotide primers and *E. coli* strains used in this study

Primers or recombinant *E. coli* strain	Relevant characteristics	Source or reference
Primers (5′–3′)		
TAUT_ins_P_K	ATACATATG**AAG**ATTGCCCAGATC	This work
TAUT_inf_P_K	ATACATATG**AAGCCT**ATTGCCCAGATC	This work
TAUT_inf_K_ins_P_K	ATACATATG**AAGAAG**CCTATTGCCCAGATC	This work
TAUTR	GATCTGGGCAATCTTCATATGTAT	This work
T7R_seq	GCTAGTTATTGCTCAGCGG	Invitrogen
Recombinant *E. coli*		
BL21(DE3)	F⁻, *omp*T, T7 promoter regulated expression	Invitrogen
BL21(4-OT)	expressing pRSET-TAUT	[17]
BL21(4-OT_K)	expressing pRSET-TAUT_K	This work
BL21(4-OT_KP)	expressing pRSET-TAUT_KP	This work
BL21(4-OT_KK)	expressing pRSET-TAUT_KK	This work

For culture propagation, Luria–Bertani (LB) or M9 minimal medium was used.[19] M9 medium was supplemented with casamino acids (5 g L^{-1} and 50 µg mL^{-1} of ampicillin was

routinely used to select for ampicillin resistance (amp^R). Stock cultures of all strains were maintained at –80 °C in LB with glycerol (200 g L^{-1}).

Biocatalyst preparation and biocatalytic reactions

The whole-cell biocatalyst system containing wild type or mutated 4-OT was prepared as described previously, using M9 minimal medium supplemented with casamino acids (5 g L^{-1}) and ampicillin (50 µg mL^{-1}).[17] Once the culture had reached an optical density of 0.5 (600 nm; spectrophotometer Ultrospec 3300pro, Amersham Biosciences, Little Chalfont, United Kingdom), the cells were induced with 0.1 mM IPTG at 28 °C. After 12 h expression, the cells were harvested by centrifugation at 5000*g* for 10 min in an Eppendorf 5804R bench top centrifuge. The wet cell pellets were resuspended to a concentration of cell dry weight (*CDW*) of 5 g L^{-1} (*OD* = 20) in 20 mM Na$_2$HPO$_4$ buffer, pH 7.2.

Unless otherwise stated, nitroalkenes **1**–**3** were added sequentially as described previously[17] to a final concentration of 2 mM (18 mg) from a 200 mM stock solution in ethanol (**1**) or 2-butanol (**2** and **3**) and isobutanal was added to a final concentration of 20 mM. Samples (100 µL) were withdrawn from the reaction over time, centrifuged at 13000*g* for 5 min and the supernatants were analyzed spectrophotometrically as previously described, following the depletion of **1** by reduction of absorbance at 320 nm (ε = 14.4 mM^{-1} cm^{-1}).[5] Depletion of substrates **2** and **3** were monitored by TLC.[11] The products were extracted and purified from the reaction mixture with ethyl acetate (2×20 mL). The combined organic extract was washed with brine (20 mL), and dried over anhydrous MgSO$_4$. After filtration and removal of the solvent under reduced pressure, the residue was purified by dry flash column chromatography (silica gel), eluting with petroleum ether–ethyl acetate mixture in a gradient (from 7:3 to 9:1) to obtain the pure nitroaldehydes.

Organocatalysis using L-lysine lithium salt

The general procedure for the synthesis of γ-nitroaldehydes from the corresponding aldehydes catalysed by L-lysine lithium salt (LysOLi) was realised using reported procedures.[20,21]

Analytical methods (HPLC) and characterization of γ-nitroaldehydes

The NMR spectra were recorded on a Varian Gemini 200 (^{1}H-NMR at 200 MHz, in deuterated chloroform). The NMR data are given in the Supplementary material to this paper. The chemical shifts are expressed in ppm (δ) using tetramethylsilane as an internal standard; the coupling constants (*J*) are in Hz.

RESULTS AND DISCUSSION

Previously, a whole-cell system based on recombinantly expressed 4-OT originating from *Pseudomonas putida* mt-2 was described and shown to be an effective biocatalyst for asymmetric Michael addition of acetaldehyde to β-nitrostyrene.[17] In addition, the biocatalytic strategy was extended by showing that this biocatalyst also accepts various substituted aromatic and heterocyclic nitroalkenes as acceptors and branched aldehydes as donors.[11] The aim was to investigate the influence of substitution of the Pro1 of 4-OT with basic amino acid(s) lysine and/or arginine, having reactive ε-amino groups that could make enamines with branched aldehydes, on the efficiency of the Michael-type reaction (Scheme 2). Moroz and co-workers reported that the introduction of a highly reactive

residue into calmodulin, which does not have catalytic activity, converts its 74-residue-long C-terminal domain into an efficient esterase.[23]

Protein modelling

Changes to the *N*-terminal residue of 4-OT influence the catalytic activity of the enzyme since the *N*-terminus participates in the active site and enamine intermediate formation (Scheme 1A and Fig. 1A).[17] Protein modelling with *β*-nitrostyrene as substrate was performed in order to see how substitutions would influence the protein conformation. Replacing the *N*-terminal Pro1 with Lys1 partially changes the size of the binding pocket (Fig. 1B). The surface charge, however, remains the same since the side chain *ε*-amino group takes the position of the terminal amino group of the original proline residue due to conformational change of the *N*-terminal part of the protein. Replacing the N-terminal Pro1 with Arg1 adds more positive charge to the binding pocket, but also further shrinks the active site, influencing the conformation of the surrounding residues and blocks access to the binding pocket (Fig. 1C). One important role of lysine and arginine in proteins is the formation of electrostatic interactions, which make the protein more stable, but they could also form electrostatic interactions with other non-protein molecules.[16]
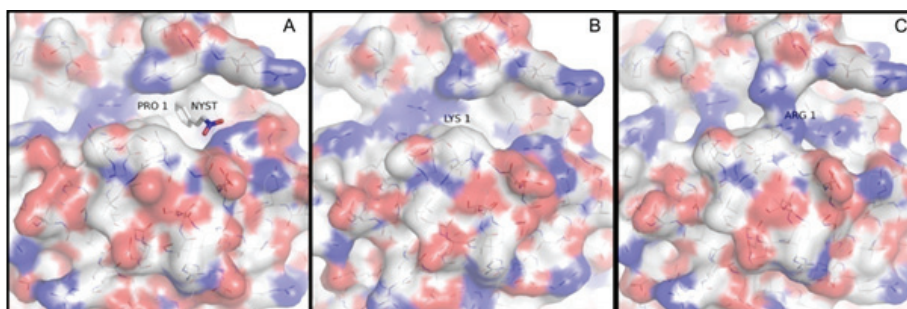

Fig. 1. Surface representation of the binding pocket with the respective *N*-terminal residue denoted; A) wild type 4-OT with docked nitrostyrene (NYST), B) 4-OT_K mutant with lysine instead of proline and C) 4-OT_R mutant with arginine blocking the access to the binding pocket.

The effect of the arginine on protein activity was shown by Sokalingam and co-workers in the mutagenesis of lysine to arginine, where they observed that this mutation affected protein folding, which decreases the productivity of functional mutants.[24] This might be a consequence of the guanidinium group of arginine, which allows interactions in three possible directions, and allows arginine to form a large number of electrostatic interactions.[16] In the case of the Arg1 mutant of 4-OT where active site is small, the arginine makes interactions that block the active site. Based on the results of protein modelling, it was decided to per-

form mutations with lysine and make three mutants, substituted Pro1 with Lys (4-OT_K), add lysine in front of the proline (4-OT_KP) and added lysine to the lysine substituted Pro1 (4-OT_KK). It is worth noting that during *in silico* analysis the possibility of the presence of the initial methionine from the recombinantly expressed protein was not taken into consideration. Methionine excision usually happens during the posttranslational tailoring of the proteins due to the presence of methionine aminopeptidases in the expression strain. This process is highly dependent on the penultimate *N*-terminal residue of its substrate. The results obtained show that the extent of excision decreased with increasing size of the introduced second amino acid.[24–27]

Organocatalytic reactions with substrates 1–3

Organocatalytic reactions were conducted with LysOLi as the catalyst and control reactions with phenylalanine lithium salt (PheOLi) as the catalyst. The reaction mixtures were stirred for 5 days at room temperature for organocatalysis with Lys, while a shorter time was needed when Phe was used as the catalyst. The asymmetric organocatalytic approach with the lithium salt of lysine and CH_2Cl_2 as the reaction medium yielded 21, 54 and 5 % of products **4**–**6**, respectively (Table II). In comparison with the control reactions with PheOLi, the yields of the products with LysOLi were lower, except when **3** was used as the substrate, when LysOLi yielded 5 % of product **6** while no reaction occurred with PheOLi. Considering that lysine is an *α*-amino acid and has an *ε*-amino group that often participates in hydrogen bonding and as a general base in catalysis, this amino acid could easily form an enamine species with branched aldehydes.

TABLE II. Yield of reaction products **4**–**6** obtained with organocatalysis and biocatalysis with wild-type 4-OT and terminal lysine mutants

Product	Organocatalysis yield, mg		Biocatalysis yield, mg			
	LysOLi	PheOLi	4-OT_K	4-OT_PK	4-OT_KK	4-OT_wt
4	5.6	13.86	9.33	8.76	7.69	15.93
5	16.57	23.93	6.44	15.95	17.18	10.43
6	1.36	NA	2.99	4.64	7.36	4.91

This might be the reason why the reaction occurred with substrate **3**, although the product yield was very low, while no reaction was observed when Phe was used as the catalyst. Lower product yield when **1** and **2** were used as substrates in the reactions with LysOLi could be attributed to the structural differences of the substrates.

Biotransformation of 1–3 with 4-OT wild type biocatalyst and 4-OT lysine mutants

All biocatalytic reactions were performed with the wild-type and the three lysine mutants with **1**–**3** as the Michael acceptors and isobutyraldehyde as the

Michael donor. All reactions were performed for 18 h, except in the case with wild type 4-OT biocatalyst and β-nitrostyrene (**1**), when the reaction was complete within 4 h.
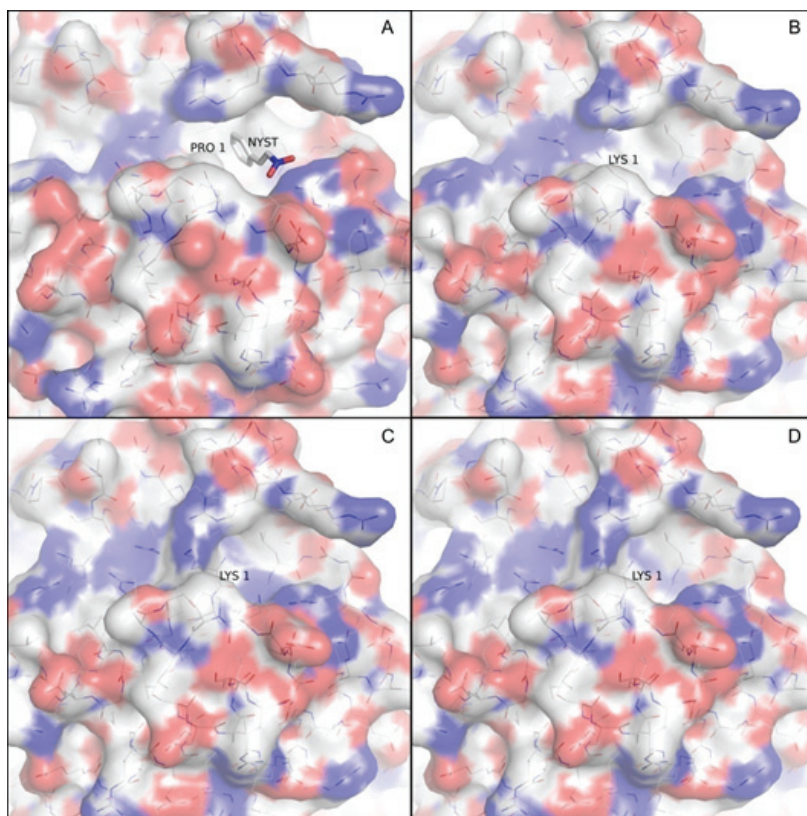

Fig. 2. Surface representation of the binding pocket of the wild type and lysine mutants; A) wild type with docked nitrostyrene (NYST), B) 4-OT_K mutant , C) 4-OT_PK mutant and D) 4-OT_KK mutant.

When β-nitrostyrene (**1**) was used as the substrate, the wt biocatalyst performed the best because the overall reaction time was 4.5 times shorter than the reactions when mutants were used as biocatalysts. In addition, the product yield was around 2 times greater when wt 4-OT was used as the biocatalyst (Table II). However, in comparison with the organocatalytic reaction with the lithium salt of lysine, biocatalysis was better considering the reaction time and product yield, showing 6 and 1.5 times improvement, respectively. In the case of substrate **1**, all mutations had a negative effect on the enzyme activity. The effect of the generated mutations was assessed by protein modelling experiments in which β-nitrostyrene was used as the substrate. Replacing the *N*-terminal proline with lysine

partially changes the size of the binding pocket (Fig. 2B). Adding another lysine residue to the *N*-terminus of the polypeptide chain significantly shortens the size of the binding pocket, thereby influencing possible interaction of ligands with Arg39 [28] but keeping the net positive charge of the binding pocket (Fig. 2C). Mutation of Pro1 to Lys1 and addition of lysine to the *N*-terminus had obvious consequences on the binding pocket by adding a positive charge to the previously hydrophobic bottom of the active site (Fig. 2D), thereby influencing the binding behaviour of the ligands. In addition, the added *N*-terminal lysine (as is the case with 4-OT_PK) decreases the active site cavity and disturbs possible interactions with Arg39.

Although, lysine is found frequently in active sites because of its positively charged ε-amino group that can form electrostatic interactions,[16] this mutation had a negative effect on the activity of 4-OT in reactions with substrate **1**. However, the reaction still proceeded indicating that Pro1 is important, but not essential for the 4-OT activity in the Michael-type addition of isobutanal to β-nitrostyrene. This is also supported by previously obtained results on the *N*-terminal proline-enriched mutants, namely 4-OT_P and 4-OT_2P, because the activity of the 4-OT_2P mutant was lower, while 4-OT_P had a similar activity to wild type 4-OT, when **1** was used as the substrate.[17]

When (*E*)-1-nitro-2-(2-thienyl)ethene (**2**) was used as the Michael donor, the least efficient was the 4-OT_K mutant yielding 21 % of product **5**. The organocatalyst LysOLi and the mutants 4-OT_PK and 4-OT_KK showed similar efficiency, yielding 54, 52 and 56 % of product **5**, respectively, while wt 4-OT yielded 34 % of product **5** (Table II). The lysine mutations had a positive influence on the biocatalyst activity when **2** was used as the Michael acceptor.

In the case when *trans*-*p*-chloro-β-nitrostyrene (**3**) was used as the substrate, the lowest amount of product **6** was obtained in the organocatalysis with LysOLi (5 %), while in the reaction with PheOLi, no product could be isolated. It was shown that electron-withdrawing substituents at the aromatic ring of β-nitrostyrene are poor Michael acceptors and influence the reaction time.[17] The 4-OT_KK mutant performed the best in comparison to reactions when 4-OT, 4-OT_PK and 4-OT_KK were used as biocatalysts, yielding 1.5, 2.5 and 1.6 times more product **6,** respectively (Table II).

CONCLUSIONS

During this study, the effect of the substitution of the terminal proline with lysine on the promiscuous activity of 4-OT was assessed. Changing Pro1 to Lys1 was not favourable for any of the substrates tested, as the mutant 4-OT_K needed more time to perform the reactions in comparison to wild type 4-OT and the product yield was lower. An additional lysine, as in 4-OT_PK mutation, did not influence the efficiency of the biocatalyst in Michael-type reactions, because the

substrate yield indicated that this efficiency was under the influence of the nature of the substrates. In the case when 4-OT was changed from Pro1 to Lys1 and an additional lysine was incorporated (4-OT_KK mutant), there was a positive effect on an enzyme activity when substrates **2** and **3** were used, but not when substrate **1** was used. Since generated mutants showed a slight improvement in activity using substrates **2** and **3**, it could be concluded that Pro1 is important, but not essential for the promiscuous Michael-type activity of 4-OT. The results suggest that future studies should be directed towards rational redesign of proteins exhibiting promiscuous activity in order to improve or change their selectivity towards certain substrates.

SUPPLEMENTARY MATERIAL

^{1}H-NMR data for compounds **4**–**6** are available electronically from http:////www.sbd.org.rs/JSCS/ or from the corresponding author on request.

И З В О Д

ЗНАЧАЈ АМИНО-ТЕРМИНАЛНОГ ПРОЛИНА ЗА НЕСПЕЦИФИЧНУ АКТИВНОСТ 4-ОКСАЛОКРОТОНАТ-ТАУТОМЕРАЗЕ (4-ОТ)

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Реакција Мајклове адиције алдехида на нитроолефине представља ефикасну методу синтезе хиралних *γ*-нитроалдехида, прекурсора аналога хиралне *γ*-аминобутерне киселине који се користе у терапијама неуролошких обољења. Целе бактеријске ћелије са хетеролого експримираном 4-оксалокротонат-таутомеразом (4-ОТ) показале су се као веома ефикасни биокатализатори у Мајкловој адицији рачвастих алдехида на *β*-нитростирене. Циљ овог рада био је да се испита утицај замене амино-терминалног пролина аминокиселинама са реактивном *ε*-амино групом, као што су лизин или аргинин, на механизам реакције Мајклове адиције. Ефекат ових мутација је анализиран *in silico*, а потом су конструисани мутанти са лизином на амино терминусу. Тестирана је способност мутаната 4-ОТ_К, 4-ОТ_РК и 4-ОТ_КК да користе *β*-нитростирен (**1**), (*E*)-1-нитро-2--(2-тиенил)оетен (**2**) и *trans-p*-хлор-*β*-нитростирен (**3**) као Мајклове акцепторе, и изобутанал као донор. Литијумова со лизина је коришћена у органокатализи са истим супстратима ради поређења. Увођење лизина на амино терминус протеина имало је негативан ефекат на Мајклову адицију, јер су приноси производа реакција смањени. Додавање лизина утицало је на структурне промене 4-ОТ чиме је побољшана активност овог ензима када су **2** и **3** коришћени као супстрати. Сходно томе може се закључити да је терминални пролин важан за активност 4-ОТ али не и кључан.

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