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ORIGINAL RESEARCH PAPER

Biotransformation of β-keto nitriles to chiral (*S*)-β-amino acids using nitrilase and ω-transaminase

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Abstract
Objective To enzymatically synthesize enantiomerically pure β-amino acids from β-keto nitriles using nitrilase and ω-transaminase.
Results An enzyme cascade system was designed where in β-keto nitriles are initially hydrolyzed to β-keto acids using nitrilase from *Bradyrhizobium japonicum* and subsequently β-keto acids were converted to β-amino acids using ω-transaminases. Five different ω-transaminases were tested for this cascade reaction, To enhance the yields of β-amino acids, the concentrations of nitrilase and amino donor were optimized. Using this enzymatic reaction, enantiomerically pure (*S*)-β-amino acids (*ee* > 99%) were generated. As nitrilase is the bottleneck in this reaction, molecular docking analysis was carried out to depict the poor affinity of nitrilase towards β-keto acids.
Conclusions A novel enzymatic route to generate enantiomerically pure aromatic (*S*)-β-amino acids from β-keto nitriles is demonstrated for the first time.

Keywords Asymmetric synthesis · β-Amino acids · β-Keto acids · β-Keto nitriles · Molecular docking · Nitrilase · ω-Transaminase

Sam Mathew and Saravanan Prabhu Nadarajan have contributed equally to this work.

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Introduction

Aromatic β-amino acids are not abundant in nature but their production has received attention because they are used as precursors for several pharmaceutical compounds and in the formation of peptidomimetics (Weiner et al. 2010). Different enzymatic routes to produce chiral β-amino acids were developed and, among these, ω-transaminase (ω-TA) is one of the most prominent ones (Rudat et al. 2012). ω-TA is a powerful biocatalyst for aminating a wide range of substrates such as ketones, aldehydes, alcohols, α-keto acids, β-keto acids and γ-keto acids (Steffen-Munsberg et al. 2015). Unlike α- and γ-keto acids, β-keto acids are thermodynamically unstable due to spontaneous decarboxylation (Rudat et al. 2012). Therefore, employing β-keto acids as initial substrates is not a

good strategy for the asymetric synthesis of β-amino acids. A hydrolysis-amination cascade system has been used in which an esterase and lipase hydrolyzed β-keto esters and then ω-TA aminated the in situ-produced β-keto acids (Banerjee et al. 2005; Kim et al. 2007). This reaction scheme was optimized to enhance the production of β-amino acids by adjusting the amount of lipase (Mathew et al. 2016a). Recently, a reductive amination-hydrolysis cascade system was introduced to enzymatically synthesize β-amino acid from various classes of β-keto esters (Houa et al. 2016).

Enzymatic hydrolysis of nitriles can generate a wide range of amides and carboxylic acids (Ebbs 2004). Moreover, different classes of nitriles can be synthesized in a simple and cost-effective way. Zhang et al. (2015) used β-nitriles as the initial substrate to generate β-keto acids using a nitrilase (NitBJ) from *Bradyrhizobium japonicum* USDA 110 (Zhang et al. 2015). The β-keto acid generated by NitBJ was then converted to β-amino acid using β-amino acid dehydrogenase. However, in order to use β-amino acid dehydrogenase, NADPH recycling is needed which requires an additional enzyme such as glucose dehydrogenase. In this study, a novel cascade system employed NitBJ and ω-TAs which were optimized to synthesize various chiral β-amino acids using β-keto nitriles as initial substrates (Fig. 1).

Materials and methods

Chemicals

Nitriles (**1a—f**), *rac*-β-amino acids (**3a—f**), (*S*)-α-methylbenzylamine (MBA), GITC (2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosylisothiocyanate) and PLP (pyridoxal 5′-phosphate hydrate) were obtained from Sigma-Aldrich. All other chemicals were of analytical or reagent grade.

Expression and purification of enzyme

The NitBJ gene was codon-optimized and synthesized with *N*-terminal His-tag by Bioneer Corporation, South Korea (Supplementary information). The gene was then inserted into an IPTG inducible pETDuet expression vector at *BamH*I and *Hind*III restriction sites. The plasmid was then introduced into the *Escherichia coli* (BL21) and the transformants were grown at 37 °C in 1 l lysogeny broth containing 100 μg ampicillin/ml. When the OD_{600} reached 0.5–0.6, IPTG was added to 0.2 mM. After 16 h of induction at 20 °C, the over-expressed cells were centrifuged at ~4000×*g* for 20 min at 4 °C. The cell pellets were resuspended and washed twice with 20 mM Tris/HCl buffer (pH 8). The suspension was then subjected to ultrasonic disruption for 20 min at 4 °C. The cell sonicate was centrifuged at ~16,000×*g* for 30 min. The *N*-terminal His6-tagged NitBJ protein was purified at 4 °C on a Ni–NTA agarose resin obtained from Qiagen (Hilden, Germany). The expression of ω-TAs from *Polaromonas* sp. JS666 (ω-TAPO), *Burkholderia graminis* (ω-TABG), *Mesorhizobium* LUK sp. (ω-TAMes), *Variovorax paradox* (ω-TAVP) and *Sphaerobacter thermophilus* (ω-TAST) was performed as reported elsewhere (Bea et al. 2011; Mathew et al. 2015; Kim et al. 2007; Crismaru et al. 2013; Mathew et al. 2016a).

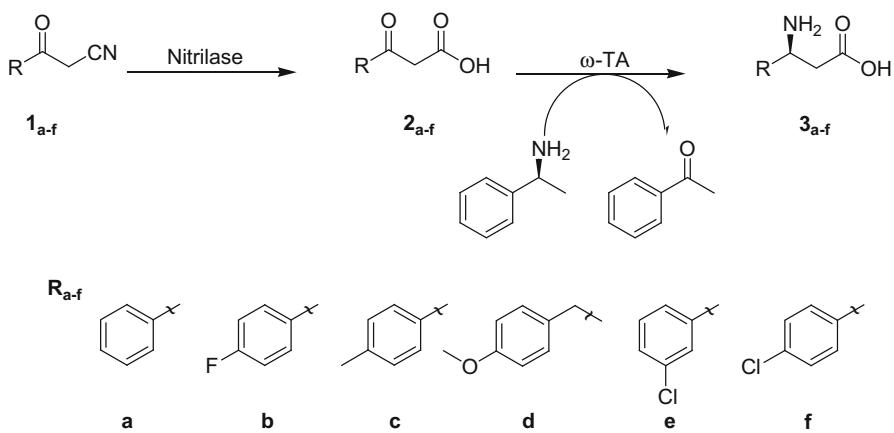

Fig. 1 Biotransformation of chiral β-amino acids from β-keto nitriles using nitrilase and ω-transaminase. Nitriles (**1a—f**) are initially hydrolyzed to β-keto acids using nitrilase and subsequently the β-keto acids (**2a—f**) are aminated using ω-transaminase to produce β-amino acids (**3a—f**)

Enzyme assay

The reaction for finding the specific activity of NitBJ was carried out in 0.5 ml at 37 °C in a Tris/HCl buffer (100 mM, pH 8.0), **1a—f** (5 mM) and NitBJ (purified-0.065 mg/ml; over-expressed *E.coli*-NitBJ 2.5 mg/ml). The enzyme reaction was stopped after 30 min by the addition of 10% (v/v) perchloric acid (50% v/v). The mixture was then centrifuged at 13,000×*g* for 15 min and the depleted substrate was subsequently analyzed. One unit of NitBJ activity is defined as the amount of enzyme that hydrolyzed 1 μmol β-keto nitrile (**1a—f**) in 1 min. The reaction for determining the specific activity of ω-TAPO was carried out in 0.5 ml at 37 °C in a Tris/HCl buffer (100 mM, pH 8.0), *rac*-**3a** (10 mM) and over-expressed *E.coli*-ω-TAPO (1 mg/ml). The reaction was stopped after 30 min by the addition of 10% (v/v) perchloric acid (50% v/v) and centrifuged as before; subsequently the depleted *rac*-**3a** was analyzed. One unit of ω-TAPO activity is defined as the amount of enzyme that deaminated 1 μmol *rac*-**3a** in 1 min. The specific activities of the ω-TAPO and NitBJ over-expressed in *E. coli* BL21 cells towards *rac*-**3a** were 0.02 and 0.014 U/mg DCW, respectively. The analytical conditions for β-keto nitriles and β-amino acids are mentioned in the supplementary information (Supplementary Tables 1, 2).

Molecular modeling and docking simulation

A sequence similarity search (BlastP) was done against protein data bank using nitrilase (Nit BJ) sequence showed only one structural template (PDB ID-3WUY) with low identity (34%) and query coverage of 91%. Generally, a model constructed using a single template with low identity can affect the robustness of the protein model. A multi-template model with defined structure restraints will provide a better model. To identify the other structural templates, the sequence was submitted to the SWISS-MODEL web-server, and more than 20 different structural templates that exhibited a similar kind of fold were identified. For secondary structure analysis, we chose the structural templates that had query coverage above 0.8 (Supplementary Table 3). Additionally, the secondary structure prediction was carried out using Psipred, Jpred. Structural restraints of secondary structure were fixed, based on the confidence level of predicted secondary structures and comparing them with retrieved structural templates. A multiple sequence alignment was performed for the NitBj protein sequences with its structural templates, NitBJ using multalin and represented with secondary structure of 3WUY using EsPript3. Subsequently, multi template modelling of was carried out using the predicted structural restraints and 3WUY, 5H8I, 1UF5 as structural templates (Zhang et al. 2014; Sekula et al. 2016). The overall structural and stereo-chemical qualities of the protein were assessed using the SAVES server and optimized for better quality using loop refinement and energy minimization of Modeller tool (Eswar et al. 2014). To identify the active site, the model structure was then superimposed with a ligand co-crystallized amido hydrolases template (PDB ID -1UF5) which depicted perfect orientation of the catalytic triad (Cys, Glu, and Lys) in the active site.

Three-dimensional structures of β-nitrile analogs (**1a—f**) were constructed using online tool–Corina 3D. Next, hydrogen atoms and Kollman charges were added to the ligands using the Python Molecular Viewer and exported for docking study as mol2 files. A docking calculation for compound **1a—f** was performed with the constructed model using Autodock4.2 (Morris et al. 2009). Prior to the docking calculations, all hydrogens and Gasteiger charges were assigned to the 3D structures of the nitrilase using the Python Molecular Viewer, and the non-polar hydrogens were added and merged. The grid size was set to 10 Å centered on the XYZ coordinate at the conserved residues (Glu46). A Lamarckian genetic algorithm was employed as a search parameter, and for each compound, a 50 GA run was carried out. For each GA run, 25,000,000 (long) evaluations were performed for a population size of 150. The operator weights for crossover, mutation and migration were set as default parameters. The best-docked conformation was selected based on the interaction (hydrogen bond interaction with Glu46, Glu135, Lys129 and Cys162), the orientation in the active site and the docking score (Auto dock score). The docked complexes of best ranking solutions were exported in PDB format for further analysis.

Results and discussion

NitBJ activity towards β-nitriles

NitBJ has been used to hydrolyze two β-keto nitriles, 5-(methylthio)-3-oxopentanenitrile and benzoylacetonitrile (**1a**), to their corresponding β-keto acids (Zhang et al. 2015). Based on this reports we chose NitBJ to hydrolyze various β-keto nitriles (**1a—f**). First, a recombinant nitrilase enzyme was sought for carrying out the cascade reaction (Fig. 1). Initially, NitBJ gene was cloned in pET24ma vector but, despite expressing the gene in different conditions, most of the enzyme was formed in the inclusion body. Subsequently, the NitBJ gene containing *N*-terminal His-tag was cloned in pETDuet vector, and (see "Materials and methods" section) the enzyme's expression was better than that of pET24ma. Under optimum conditions, a partially soluble protein was obtained, and the purified enzyme's specific activity was 0.36 U/mg.

To explore the ability of NitBJ to hydrolyze various β-nitriles, the specific activity of purified NitBJ towards **1a–f** was determined (Fig. 2a). NitBJ showed considerable activity towards all substrates, except 3-(4-bromophenyl)-3-oxopropanenitrile. Although 3-(4-bromophenyl)-3-oxopropanenitrile was included, NitBJ activity towards it could not be measured due to its poor solubility. One pattern that was noticeable was the enzyme's lower activity towards bulkier substrates. Purified NitBJ showed the highest activity towards **1a** and the least activity towards **1f**. The activity of NitBJ towards various β-keto nitriles demonstrated its potential to synthesize various β-amino acids by combining it with ω-TA.

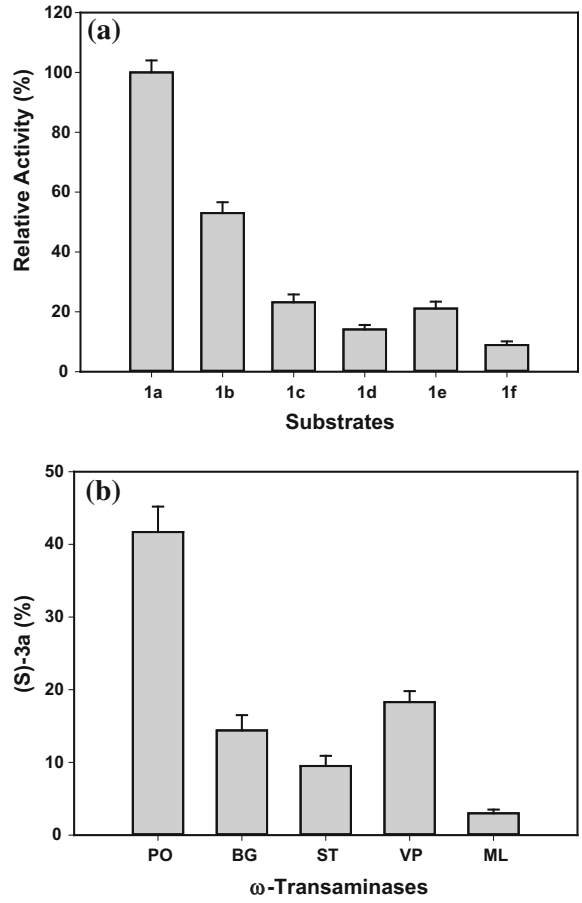

Fig. 2 **a** Substrate specificity of nitrilase. Reaction condition: Reaction volume 0.5 ml, 10 mM **1a—f** and nitrilase (0.065 mg/ml), 100 mM Tris/HCl buffer (pH 8) at 37 °C for 30 min. 100% 0.36 μmol/min. **b** Production of **3a** using nitrilase and various ω-transaminases. Reaction condition: Reaction volume 0.5 ml, 10 mM **1a**, 20 mM (*S*)-α-MBA, 0.1 mM PLP, 10% (v/v) DMSO, ω-TAs (20 mg DCW/ml), NitBJ (20 mg DCW/ml), 100 mM Tris/HCl buffer (pH 8) at 37 °C for 24 h. The *error bar* indicates the standard deviations from three individual measurements

Selection of ω-transaminase for the cascade reaction system

Five previously reported ω-TAs, that had shown activity towards aromatic β-amino acids, were overexpressed in *E. coli* BL21 cells and utilized in this study, together with NitBJ to produce (S)-β-amino acids. The crystal structures of two of those ω-TAs, ω-TAML and ω-TAVP, were described previously (Wybenga et al. 2012; Crismaru et al. 2013). Our group has reported a thermostable ω-TAST that has activity toward both aromatic β- and γ-amino acids (Mathew et al. 2016a); ω-TAPO and ω-TABG have also been reported to show activity towards both β- and γ-amino acids (Bea et al. 2011; Mathew et al. 2016b). All five ω-TAs were overexpressed in *E. coli* BL21 cells and utilized along with NitBJ to produce (*S*)-β-amino acids. The ω-TAs such as *Vibrio fluvialis* and *Chromobacterium violaceum* commonly use L-Ala as amino donor (Koszelewski et al. 2010). However, in the case of ω-TAs which have activity towards aromatic β- and γ-amino acids, (*S*)-α-MBA was reported to be a better amino donor. A previous study of the synthesis of aromatic β-amino acids

using ω-TAPO showed that (*S*)-α-MBA as the amino donor gave better yield compared to other typically used amino donors such as L-Ala, isopropylamine and benzylamine, including the pyruvate removal system employing lactate dehydrogenase (Mathew et al. 2016a). Therefore in the present study, the enzymatic reaction used (*S*)-α-MBA as the amino donor for all ω-TAs.

To select the best ω-TA, a whole cell system containing ω-TAs (10 mg DCW/ml), NitBJ (10 mg DCW/ml), 0.1 mM PLP, 10% DMSO (v/v), and 20 mM (*S*)-α-MBA was employed to synthesize (*S*)-**3a** from **1a.** All cascade reactions produced (*S*)-**3a**. The enzyme reaction using ω-TAPO gave the highest analytical yield (45%) while ω-TAML gave the lowest yield (3%) (Fig. 2b). Based on these results, ω-TAPO was selected for further enzymatic reactions with NitBJ.

Optimization of nitrilase and amino donor concentration to generate chiral β-amino acids from β-nitriles

Our group had earlier generated chiral aromatic β-amino acids by using β-ketoesters as initial substrate (Mathew et al. 2016a). In that work, optimization of the lipase concentration in the reaction had a direct impact on the final yield of β-amino acids. Therefore, to determine the optimum concentration of NitBJ in the reaction media, enzymatic reactions of **1a** in the presence of ω-TAPO (20 mg DCW/ml) and 20 mM (*S*)-α-MBA was performed with different concentrations of NitBJ over-expressed in *E. coli* BL21 cells (0–30 mg DCW/ml) (Fig. 3a).

The yield (**3a**) increased with increasing concentration of NitBJ in the reaction media. The highest yield for **3a** was observed when the nitrilase concentration was 25 mg DCW/ml. However, the yield decreased with a NitBJ concentration of 30 mg DCW/ml. This lower yield can be due to **1a** being hydrolyzed faster than **2a** is aminated, which leads to the decarboxylation of **2a** and thereby affecting the overall yield. The same phenomenon was earlier reported when lipase hydrolysis of β-keto ester was more rapid than the TA activity (Mathew et al. 2016a).

An unfavorable equilibrium is one of the major challenges while performing asymmetric synthesis. Over the years, different methods have been developed to make the equilibrium favorable to the product side. These include introducing enzymes to remove

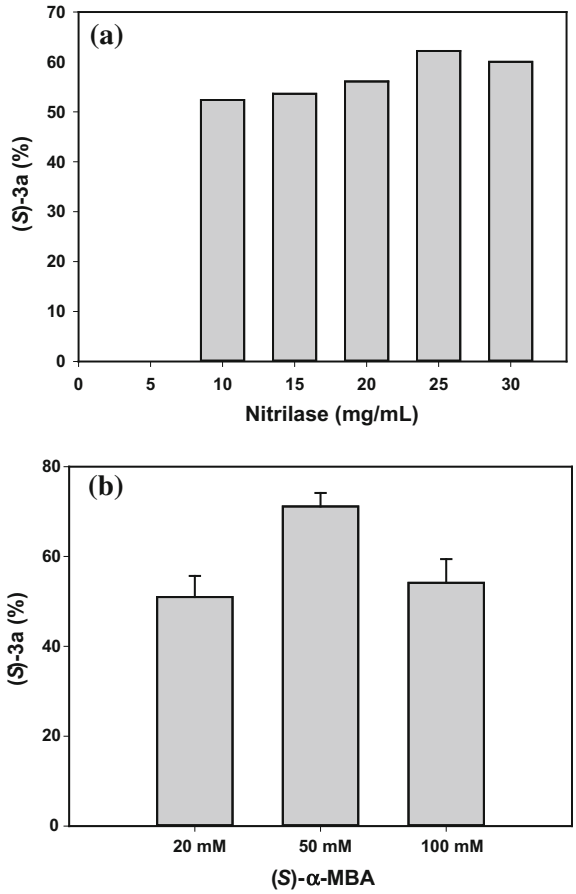

Fig. 3 **a** Asymetric synthesis of **3a** with different concentration of nitrilase. Reaction condition: Reaction volume 0.5 ml, 10 mM **1a**, 20 mM (*S*)-α-MBA, 0.1 mM PLP, 10% (v/v) DMSO, ω-TAs (20 mg DCW/ml), NitBJ (20 mg DCW/ml), 100 mM Tris/HCl buffer (pH 8) at 37 °C for 24 h. **b** Asymetric synthesis of **3a** with different concentration of amino donor. Reaction conditions: reaction volume 0.5 ml, 10 mM **1a**, 20 mM (*S*)-α-MBA, 0.1 mM PLP, 10% (v/v) DMSO, ω-TAs (20 mg DCW/ml), NitBJ (20 mg DCW/ml), 100 mM Tris/HCl buffer (pH 8) at 37 °C for 24 h. The *error bar* indicates the standard deviations from three individual measurements

by-products or using organics solvents to selectively extract the by-product (Koszelewski et al. 2010). Volatile products, such as acetone, can be removed by increasing the temperature. Increasing the concentration of amino donor is a simple and common strategy that is employed to shift the equilibrium towards the product side. However, increasing the concentration of amino donor can also adversely affect the enzyme activity due to substrate inhibition. To find the optimum amount of (*S*)-α-MBA as amino donor, enzymatic reactions with different amino

donor concentrations (20, 50 and 100 mM) were performed (Fig. 3b). As expected, the change in the amino donor concentration had a direct bearing on the final yield of the product. With the amino donor at 20 , 5.1 mM (*S*)-**3a** was generated while 7.1 mM was generated when the amino donor was at 50 mM. However, when 100 mM (*S*)-α-MBA was used it resulted in a lower yield of 5.4 mM which may due to the enzyme's inhibition towards the amino donor. Previously, ω-TABG and ω-TAST were shown to have reduced activity in the presence of high concentration of (*S*)-α-MBA (Mathew et al. 2015; Shon et al. 2014).

Based on these results, we performed further enzymatic reactions with **1b—f** using 50 mM (*S*)-α-MBA to generate (*S*)-β-amino acids (Table 1). In the case of other substrates (**1b—1f**), higher NitBJ concentration (30 mg DCW/ml) was added to the reaction mixture as NitBJ had a lower activity towards **1b—f** when compared to that of **1a**. All products showed medium to low yield but with very high purity ($ee > 99\%$). One reason for low yield can be the enzyme's poor activity towards **1b—f**. Since the low yield of β-amino acids was directly correlated to the amount of unhydrolyzed substrate, it was evident that NitBJ's activity was the bottle neck for the synthesis of β-amino acids. The narrow substrate specificity of NitBJ, when compared to that of ω-TAPO, was the main limiting factor for obtaining a good yield. An enzymatic cascade system using nitrilase and ω-TA for the synthesis of β-amino acids may improve the yield by employing a nitrilase with high activity and broad substrate specificity towards β-keto nitriles. In order to identify the residues responsible for the narrow specificity of the enzyme towards β-keto nitriles, homology modeling of NitBJ and docking studies of the enzyme with **1a–f** were performed.

Table 1 Production of various β-amino acids from β-keto nitriles using nitrilase and ω-transaminase

Product	Yield (%)	ee^S
3a[a]	72	>99
3b	63	>99
3c	46	>99
3d	8	>99
3e	49	>99
3f	22	>99

Reaction conditions Reaction volume 0.5 ml. 10 mM **1a–f**, 50 mM (*S*)-α-MBA, nitrilase (30 mg DCW/ml), ω-TAPO (20 mg DCW/ml), 0.1 mM PLP, 10% (v/v) DMSO and 100 mM Tris/HCl pH 8 at 37 °C for 24 h

All yield values are means from three individual measurements and standard deviations did not exceed 10%

[a] For the production of **3a**, nitrilase (25 mg DCW/ml) was used

Molecular interaction of β-keto nitriles with nitrilase

A three dimensional structure of NitBj was constructed using three different structural templates (PDB ID- 3WUY, 5H8I, and 1UF5) and with defined secondary structural restraints obtained from PsiPred and Jpred. (Supplementary Figs. 1, 2, 3). After evaluating the model in SAVES server, the model was refined to obtain a good quality three dimensional structure (Supplementary Figs. 4, 5) After validating the quality of final model, the active site of NitBJ is predicted by superimposing the modeled structure with the structure template co-crystallized with its substrate (PDB ID-1UF5). Based on structural superimposition, the active site of nitrilase is comprised of amino acids such as a Glu46, Tyr52, Leu111, Lys129, Thr132, His133, Glu135, Cys162, Ala163, Trp186, Pro187, Ser188, Thr190, Leu191, Leu199, Gly200, Val203, and Asn204. The model structure was then docked with the β-keto nitrile substrates (**1a—f**) at Glu46 residue (catalytic triad residues) using Autodock4.2. The main interaction to be considered for the recognition of nitrile compounds are interaction with the catalytic triad Cys162, Glu46 and Lys129 residues. Therefore, the docked complex forming hydrogen bond interaction with one of the residue Cys162, Glu46 and Lys129, was considered as preferred binding mode. The substrate forming hydrogen bond with one of these residues was exported and interaction were analyzed (Fig. 4). Very few docked complexes show the exact binding mode in the cluster. Based on the exported complexes, the key interaction for the binding of β-keto nitriles was sketched as a 2D diagram (Fig. 4).

These results clearly show that recognition of the β-keto group of nitrile compounds forms a close interaction with His133 and Thr132 of the active site (Figs. 4, 5). The docking simulation results are consistent with the experimental results. Compound **1a** had a better binding energy compared to the other

keto nitrile analogs. On the other hand, the side chains at para position form hydrophobic interaction with residues such as Leu191, Gly200, Val203, Asn204, Leu199 (Fig. 4). The fluoro-keto nitrile analog showed a binding energy similar to compound **1a**, and it formed a close interaction with Leu191, Gly200 and Asn204. However the chloro and o-methyl analogs formed steric hindrance with Gly200 and Leu191, thereby reducing the binding energy. This demonstrates that the increase in bulky atoms at the *para*-position of the β-keto nitriles showed a poor binding score, which in turn demonstrated the steric clash between the bulkier atoms and the residues such as Leu 191, Gly200, Val203, Asn204, Leu199 (Supplementary Table 4).

From this study, it is clear that the compounds **1a** and **1b** are the better substrates compared to **1c**—**f**. Increase in the size 56 atom at the *para*-position showed a decrease in binding energy and relative activity. This demonstrates that the binding energy well correlated with the experimental results (Fig. 2a; Supplementary Table 4). Based on the docking score, the methyl-keto analog also a better substrate for the nitrilase compared to the bulky side chain containing

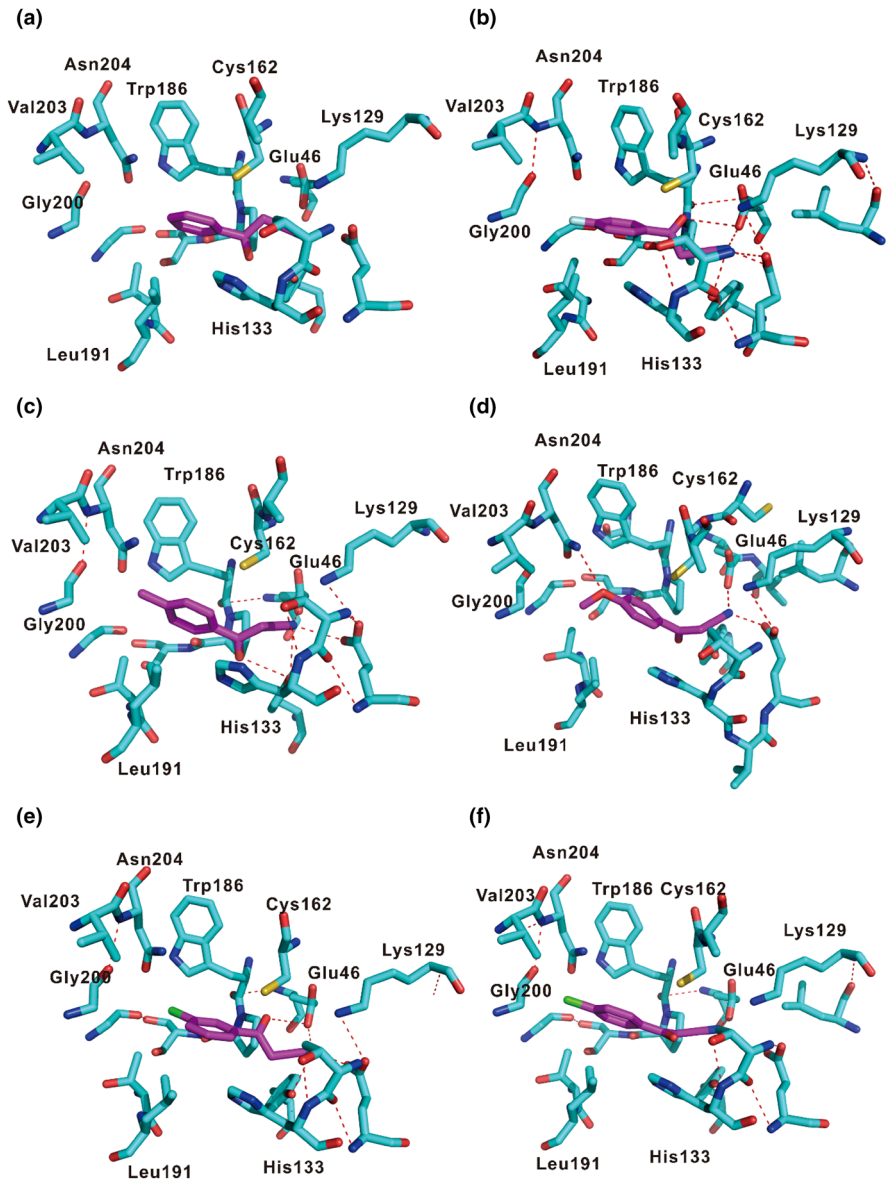

Fig. 4 Interaction of β-keto nitriles (**a**) **1a**, (**b**) **1c**, (**c**) **1d**, (**d**) **1d**, (**e**) **1e** (**f**) **1f** with the active site residues of nitrilase. The *cyan* color stick represents the active site residues, *magenta* color stick represents the substrates, *green* color represents chlorine atom, *light blue* color represents fluorine atom, *red* represent the oxygen atom and *blue* represent the nitrogen atom

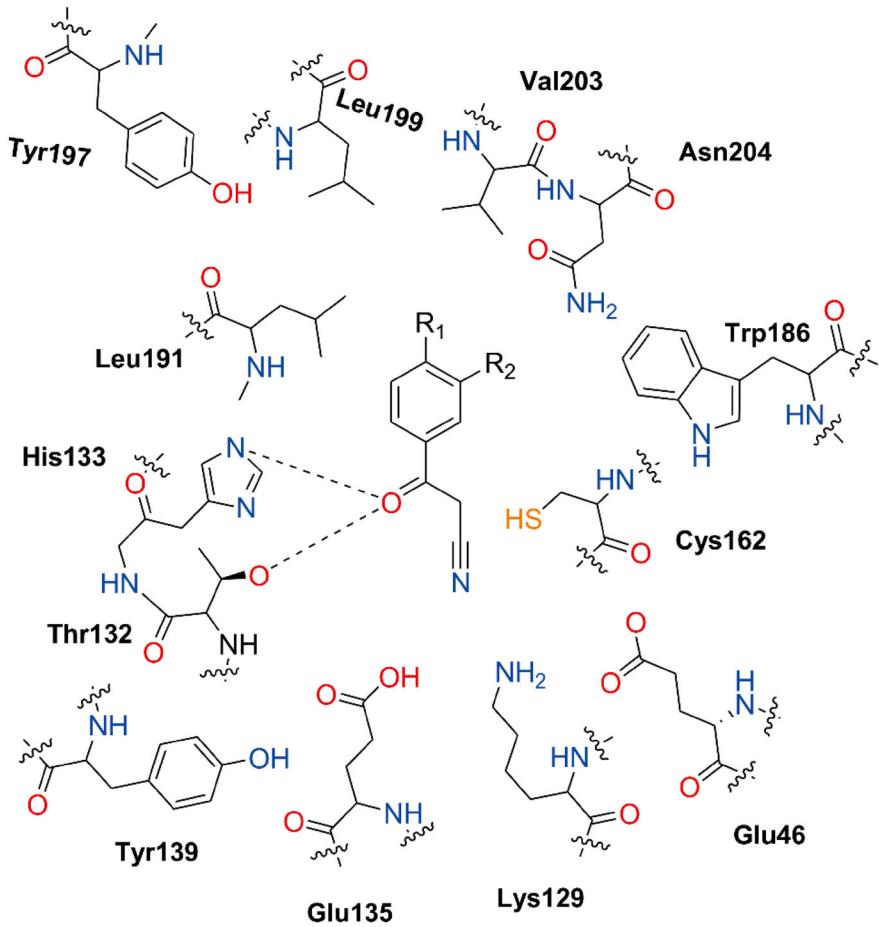

Fig. 5 Active site architecture of nitrilase with the β-keto nitrile substrates based on the docked complexes. *Red* color represents the oxygen atom, *blue* represent the nitrogen atom. R1—sidechain at *para*-position and R2—atom at *meta*-position. *Dotted line* represents key residues needed for the recognition of β-keto nitriles

analogs such as *p*-chloro, and *p*-bromo (Supplementary Table 4). Further, the bromo-containing nitrile analogs show a negligible value for the similar pose of docked complex. In the case the *o*-methyl nitrile analog, an additional interaction with the Asn204 was observed which might be the reason for enhancement in its binding affinity (Fig. 4d). The *p*-chloro-keto nitriles had steric clashes with the residues Leu199 and val203. Further, superimposition of the all the docked complexes indicates that the selectivity of the enzyme depends mainly on the active site interaction with β-ketonitriles and shows that steric clash between bulky atom and residues Leu191, Gly200, Val203, Asn204, Leu199 (Figs. 4, 5). Furthermore, the chloro side-chain at the *meta*-position of β-ketonitriles (**1e**) shows steric hindrance with Asn204 and Trp186, thereby exhibiting poor binding energy (Fig. 4e). Although, this analog (**1e**) shows a better relative activity compared to **1d** and **1f**, it shows poor binding energy. This might be due to the conformational change in active site residue Trp186 during homology modeling. Based on these docking results, it is clear that the β-ketonitriles are poor substrates for nitrilase. To increase the binding efficiency of bulkier nitrile compound, it is better to engineer the active site residues such as Asn204, Val203, Trp186 with smaller amino acid that will reduce the steric clash and enhance binding. From this analysis, it is clear that the β-ketonitriles analogs are poor substrate (**1b**—**f**) for NitBJ compared to **1a**.

Conclusions

The suitability of generating various enantiomerically pure (*S*)-β-amino acids (*ee* > 99%) using nitrilase and ω-TAs is demonstrated for the first time. In this enzymatic cascade reaction, nitrilase was utilized to hydrolyze β-keto nitriles to β-keto acids, and then ω-TA is used to aminate β-keto acids to produce chiral (*S*)-β-amino acids. Using a computational approach,

we depict the interactions of various β-keto nitriles in the active site of NitBJ and found that β-keto nitrile (**1a**) was the best substrate compared to other β-keto nitrile analogs. Engineering the active site residues such as His133, Thr132, Leu191, Tyr197, Val203 and Asn204 of NitBJ might enhance the enzyme's activity towards bulky β-keto nitriles.

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Supporting information Methods—a—Codon optimized nucleotide sequence of nitrilase in pETDuet vector; b—Analytical conditions.

Supplementary Table 1—Retention time of β-ketonitriles.

Supplementary Table 2—Retention time of β-amino acids.

Supplementary Table 3—Structural templates of nitrilase homologs with PDB ID, sequence identity and query coverage and structure resolution.

Supplementary Table 4—Binding energy of the β-keto nitrile substrates (**1a–f**).

Supplementary Fig. 1—Jpred prediction results for nitrilase proteins sequence.

Supplementary Fig. 2—PSIPRED prediction results for Nitrilase proteins sequence.

Supplementary Fig. 3—Sequence alignment of the nitrilase with its structural templates (PDBID-3WUY, 5H8I and 1UF5) using Multalin and Espript3.

Supplementary Fig. 4—Homology modeled nitrilase and protein sequence of Nitrilase.

Supplementary Fig. 5—Ramachandran plot of the homology modeled nitrilase.

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