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Driving Transamination Irreversible by Decomposing Byproduct α-Ketoglutarate into Ethylene Using Ethylene-Forming Enzyme

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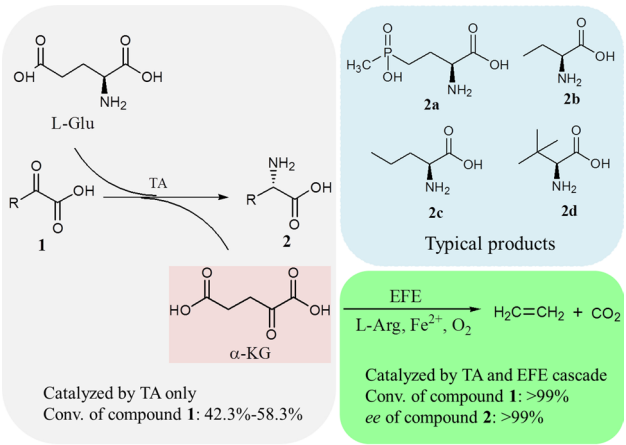
Abstract

The transformations of transaminases have been extensively studied as an approach to the production of chiral amino moieties. However, the low equilibrium conversion of the reaction is a critical disadvantage to transaminase application, and a strategy for shifting the reaction equilibrium is essential. Herein, we have developed a novel method to effectively prevent the reversibility of transamination by fully decomposing byproduct α-ketoglutarate into ethylene and carbon dioxide in situ using ethylene-forming enzyme (EFE). Two transaminases and one EFE were expressed in *E. coli* and purified to be used in the cascade reaction. After optimal reaction conditions were determined based on the enzymatic properties, a cascade reaction coupling transaminase with EFE was conducted and showed high efficiency in the synthesis of L-phosphinothricin. Finally, using this approach with only an equivalent amount of amino donor L-glutamate increased the conversions of various keto acids from < 60% to > 99%. This strategy shows great potential for transamination using glutamate as the amino donor.

Graphical Abstract



TA: transaminase; EFE: ethylene-forming enzyme; Conv.: conversion; *ee*: enantiomeric excess

Keywords Transaminase · Ethylene-forming enzyme · Reaction equilibrium · Asymmetric synthesis · Amino acids

1 Introduction

Transaminases (TAs, EC 2.6.1.X) catalyze the transfer of an amino group from a donor substrate to a ketone acceptor using pyridoxal 5′-phosphate (PLP) as cofactor (Scheme 1). TAs have been widely used to produce chiral amino moieties, including amino acids, chiral amines, amino alcohols,

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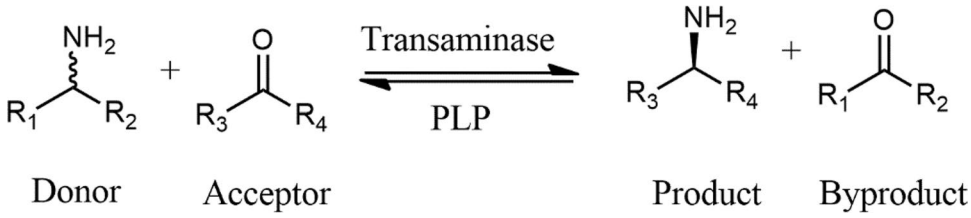
Scheme 1 General reaction scheme for transaminase

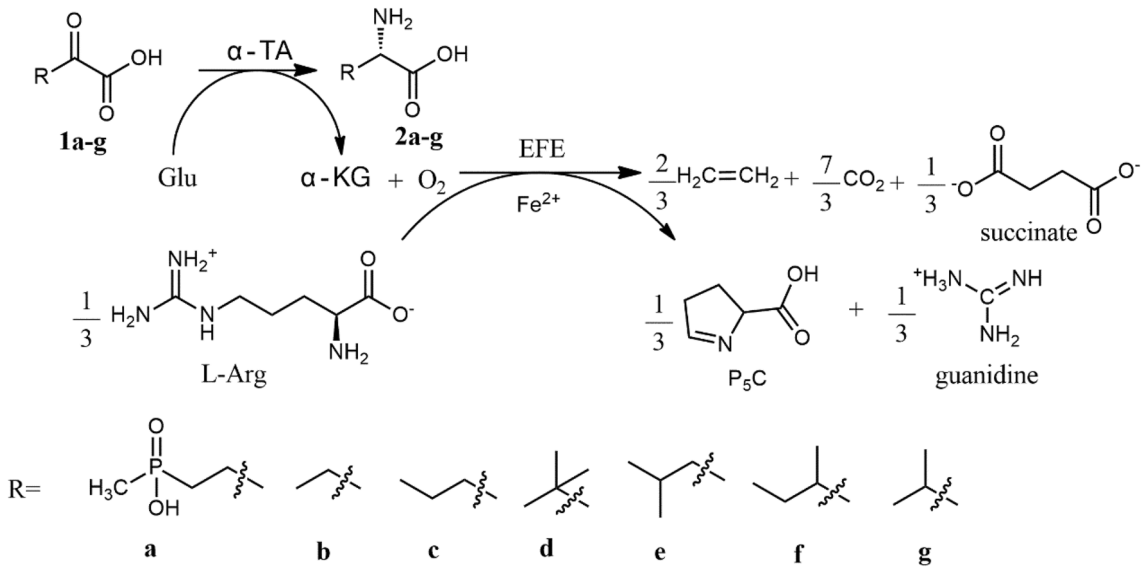


and amino sugars, which are valuable key intermediates or starting materials in the synthesis of chiral drugs and agricultural products [1–4].

However, the unfavorable thermodynamic equilibrium of transamination is a significant obstacle to the application of TAs [5–7]. For α-TAs, L-glutamate (L-Glu) can be used as a universal amino donor, with transamination typically ending at a thermodynamic equilibrium with an equilibrium constant of $K \approx 1$ [8]. For example, the equilibrium constant (K) for transamination between trimethylpyruvic acid and L-Glu is 0.67, meaning that only 45% of trimethylpyruvic acid can be converted into L-*tert*-leucine using an equivalent amount of L-Glu [9].

Several attempts have been made to shift this neutrally positioned equilibrium towards product formation, including using a stoichiometric excess of amino donor and in-situ product/byproduct removal [10]. A four-fold molar excess of L-Glu with a keto acid was used to produce L-phosphinothricin in more than 90% yield [11]. L-Homophenylalanine was produced in 94% yield owing to its poor solubility, which resulted in in-situ product precipitation [12, 13]. Furthermore, A 92% yield of L-2-aminobutyrate was achieved by recycling the α-ketoglutarate (α-KG) byproduct back to L-Glu in situ [14–18]. These strategies all effectively shifted the equilibrium to some extent.

While investigating α-KG catabolism, we found that an ethylene-forming enzyme (EFE) can decompose α-KG into ethylene and carbon dioxide with high specificity for α-KG [19–21]. This indicated that α-KG generated in the transamination reaction could be decomposed simultaneously by adding an EFE to TA in a single reaction vessel. Accordingly, we have developed a novel approach to prevent reverse transamination through in-situ decomposition of byproduct α-KG using EFE. This cascade can reach a theoretical 100% keto acid conversion. Several typical keto acids were selected as amino acceptors to verify this novel concept in transaminase-catalyzed asymmetric synthesis and demonstrate its versatility (Scheme 2).



Scheme 2 α-TA reactions for the asymmetric synthesis of chiral α-amino acids driven to completion by coupling with an ethylene-forming reaction. **1a** 2-Oxo-4-[(hydroxy)(methyl)phosphinoyl]butyric acid; **1b** 2-oxobutyric acid; **1c** 2-oxovaleric acid; **1d** trimethylpyruvic acid; **1e** 4-methyl-2-oxopentanoic acid; **1f** 3-methyl-2-oxopentanoic acid; **1g** 3-methyl-2-oxobutanoic acid; **2a** L-phosphinothricin; **2b** L-2-aminobutyrate; **2c** L-norvaline; **2d** L-*tert*-leucine; **2e** L-leucine; **2f** L-isoleucine; **2g** L-valine

2 Materials and Methods

2.1 Reagents, Strains, and Plasmids

PrimeSTAR Max DNA polymerase and restriction endonucleases were purchased from Takala (Dalian, China). Yeast extract, tryptone, antibiotics, and isopropyl β-D-1-thiogalactopyranoside (IPTG) were purchased from Sangon Biotech Co., Ltd. (Shanghai, China). Compound **1a** (Scheme 2) was synthesized and purified in our laboratory according to previous reports [22, 23]. Other chemical reagents were of analytical grade and obtained from standard commercial sources.

Escherichia coli W3110 was maintained in our laboratory and used to amplify TA genes, including branched-chain amino acid TA (BCTA, GenBank: CAQ34114.1) and 4-aminobutyrate-α-KG TA (GabT, GenBank: WP_001087611.1). *E. coli* BL21 (DE3) was used for gene expression (Novagen, USA).

Vector pET-28a (+) (Novagen, USA) was used to construct recombinant vector for gene expression. Recombinant vector pET-28-EFE was obtained from Sangon Biotech Co., Ltd. (Shanghai, China), where the gene of EFE from *Pseudomonas syringae* pv. *phaseolicola* PK2 (Genbank: D13182.1) was synthesized and inserted between *Eco*RI and *Hin*dIII restriction sites of vector pET-28a(+). Recombinant vectors pET-28-GabT and pET-28-BCTA were constructed in our laboratory (Fig. 1).

2.2 Construction of Recombinant Vectors

Genes *gabT* and *bcta* were amplified by polymerase chain reaction (PCR) using genomic DNA of *E. coli* W3110 as the template. The primers and restriction endonucleases were 5′-CCGGAATTCATGAACAGCAATAAAGAGTTA ATG-3′ (*Eco*RI) and 5′-CCCAAGCTTCTACTGCTTCGC CTCATCAAAAC-3′ (*Hind*III) for *gabT*, and 5′-CCGGAA TTCATGACCACGAAGAAAGCTGATTAC-3′ (*Eco*RI) and 5′-CCGCTCGAGTTATTGATTAACTTGATCTAA CCAGC-3′ (*Xho*I) for *bcta*. The PCR products were analyzed using 1% (w/v) agarose gel electrophoresis. Next, *gabT* and *bcta* were digested using their respective restriction endonucleases. The digested DNA fragments were then inserted into the same restriction sites of the pET-28 a(+) expression vector under the control of the T7 promoter. The constructed recombinant plasmids were pET-28-GabT and pET-28-BCTA.

2.3 Expression of Recombinant Enzymes

Recombinant *E. coli* was cultivated at 37 °C in Luria–Bertani medium with kanamycin (50 μg/mL). The cells were induced for 16 h using IPTG (0.2 mM) at 25 °C (for GabT) or 18 °C (for BCTA and EFE), at which point the OD_{600} had reached approximately 0.6. The cells were then harvested by centrifugation (8000×*g*, 5 min, 4 °C), washed with phosphate buffer (100 mM, pH 7.4), and resuspended in half the volume of buffer. The cell suspension was subjected to ultrasonic cell disruption to obtain cell lysate.

The expression of recombinant protein was examined using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Samples for SDS-PAGE were prepared in two steps. First, cell lysate was centrifuged (12,000 rpm, 5 min, 4 °C) and the supernatant was collected and stored below 4 °C, while the sediment was resuspended in phosphate buffer. Both the supernatant and resuspended sediment were then added into loading buffer, followed by denaturation at 99 °C for 10 min.

2.4 Purification of Recombinant Enzymes

The harvested cells were resuspended in equilibration buffer (20 mM phosphate buffer, 500 mM NaCl, 50 mM imidazole, pH 7.5) and lysed using ultrasonication. The supernatant (crude enzyme) was then loaded onto a Ni–NTA (nitrilotriacetic acid) chelating affinity column equilibrated with equilibration buffer. The column was eluted with a stepwise gradient of imidazole (50–500 mM) in 15–20 column volumes of equilibration buffer. Fractions containing the eluted product were identified by SDS-PAGE. The protein was then

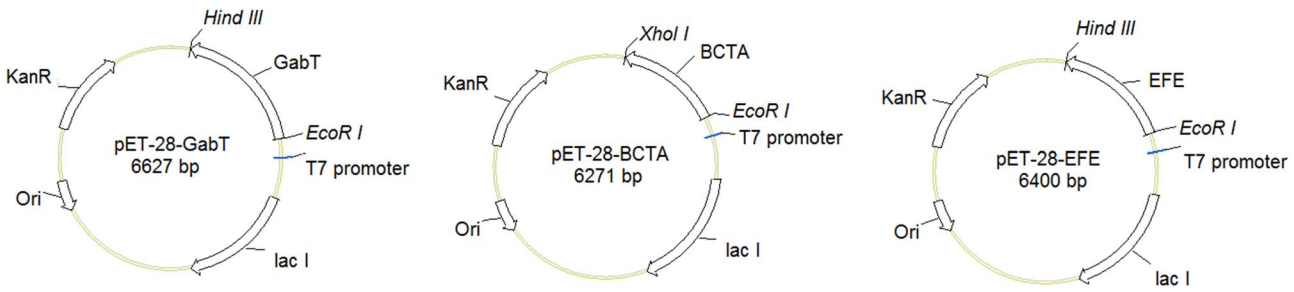


Fig. 1 Vector map for recombinant vectors expressing GabT, BCTA and EFE

desalted and replaced with phosphate buffer (100 mM, pH 7.4) by ultrafiltration.

2.5 Activity Assays and Biocatalytic Reaction

The assay solution for TA activity measurements contained phosphate buffer (50 mM, pH 8.0), PLP (1 mM), L-Glu (100 mM), and **1a** (50 mM, adjusted to pH 7 with NaOH; Scheme 2). The reaction was initiated by adding TA and allowed to react for 15 min at 45 °C. The enzyme reaction was then quenched by adding 1 M HCl and the amount of α-KG was detected by HPLC. The TA activity was defined as the amount of enzyme that catalyzed the formation of 1 μmol of α-KG from L-Glu per minute.

The assay solution for EFE activity measurements contained phosphate buffer (50 mM, pH 7.4), $FeSO_4$ (1 mM), L-arginine (L-Arg, 5 mM), and α-KG (5 mM). The reaction was initiated by adding EFE and allowed to react for 10 min at 25 °C. The enzyme reaction was then quenched by adding 16% (w/v) trichloroacetic acid. The decrease in the amount of α-KG was detected through derivatization with 2,4-dinitrophenylhydrazine using a colorimetric method [24]. The EFE activity was defined as the amount of enzyme that catalyzed the consumption of 1 μmol of α-KG per minute.

Biocatalytic reactions were performed in 15 × 150 mm test tubes at 25 °C and 220 rpm using a constant temperature shaker. Other reaction conditions are shown in the respective tables.

2.6 Analysis

The keto acids (α-KG and compounds **1a**–**g** in Scheme 2) were detected by analytical HPLC using a reverse-phase C18 column (Ultimate AQ-C18, 5 μm, 4.6 × 250 mm). The flow rate was maintained at 1 mL/min with detection at 205 nm, and the column temperature was maintained at 40 °C. The mobile phase was composed of buffer A (50 mM $(NH_4)_2HPO_4$ and 0.1% (w/v) tetrabutylammonium hydroxide, adjusted to pH 3.6 with 50% (w/v) phosphoric acid) and acetonitrile. α-KG, **1a, 1b**, and **1 g** were eluted with buffer A/acetonitrile (90:10, *v*/*v*), while **1c**–**f** were eluted with buffer A/acetonitrile (80:20, *v*/*v*).

The concentration and enantiomeric excess (*ee*) of the amino acids (L-Glu and products **2a**–**g** in Scheme 2) were determined by analytical HPLC using a C18 column (Pntulips QS-C18, 5 μm, 4.6 × 250 mm) after derivatization with *o*-phthalaldehyde and *N*-acetyl-L-cysteine [25]. The flow rate was maintained at 0.85 mL/min, with detection at 338 nm, and the column temperature was constant at 30 °C. L-Glu and **2a** were eluted with 50 mM sodium acetate buffer/acetonitrile (95:5, *v*/*v*), **2b** and **2 h** were eluted with 50 mM sodium acetate buffer/acetonitrile (85:15, *v*/*v*), and **2c**–**2e** and **2 g** were eluted with 50 mM sodium acetate buffer/acetonitrile (80:20, *v*/*v*).

3 Results and Discussion

3.1 Establishment of Cascade Reaction Conditions

Initially, the feasibility of the cascade reaction in one-pot was assessed based on the compatibility of the involved enzymes. GabT has been reported to exhibit a maximum reaction velocity at 55 °C and pH 8.0–9.5 [11], while insufficient information exists on the optimal reaction conditions for BCTA, although BCTA-catalyzed reactions have been indicated to take place at 37 °C and pH 7.5–8.0 [26]. The highest activity of EFE from *Pseudomonas syringae* pv. *phaseolicola* PK2 has been reported at 20–25 °C and pH 7.0–7.5 [19, 27].

These three enzymes were cloned and expressed in *E. coli* BL21 (DE3) and then purified to electrophoretic purity (see Figs. S1 and S2). The specific activities of GabT and BCTA were 65.5 ± 1.1 and 41.3 ± 2.0 U/mg, respectively, when using **2a** as a model product. However, the specific activity of EFE was 0.14 ± 0.04 U/mg, which was significantly lower than that of the TAs.

To select the optimal reaction conditions for the cascade reaction, the enzymatic properties of the three recombinant enzymes were characterized. The results indicated that both GabT and BCTA were more active and stable than EFE at high temperatures and pH levels, while EFE retained its high activity and stability at 25–30 °C and pH 7.0–7.5 (Table 1). Due to EFE having a much lower specific activity than TAs,

Table 1 Enzymatic properties of three enzymes involved in this study

Enzymes	GabT	BCTA	EFE
Optimal reaction temperature	75 °C	75 °C	30 °C
Optimal reaction pH	pH 9.0–11.0	pH 6.0–12.0	pH 7.5
Thermal stability	72.7 °C[a]	67.9 °C[a]	Stable below 25 °C
pH stability	Stable at pH 6.0–12.0	Stable at pH < 10.0	Stable at pH 7.0–7.5

See Figs. S3 and S7

[a]Thermal stability is indicated by the kinetic thermal temperature, which means that the residual activity was 50% when incubated at this temperature for 15 min

the reaction conditions of the cascade were set to pH 7.5 and 25 °C, because EFE performed better under these conditions while the TAs still showed high activities (Figs. S3 and S7).

3.2 Effect of TA and EFE Cascade on the Conversion of 1a

The effect of coupling EFE with TA on the reaction equilibrium position was first evaluated by converting **1a**. L-Arg was added to the reaction system because it was essential for α-KG decomposition, but did not serve as an amino donor for TAs [27–30]. Due to its rather low specific activity, large amounts of EFE were added to reduce the reaction time. However, the decomposition of α-KG catalyzed by EFE remained the rate-limiting step in the cascade reaction. The results showed that, when TA was coupled with EFE, byproduct α-KG was fully decomposed and the conversion of **1a** (Scheme 2) was improved from 55.6% to over 99% using only one equivalent of amino donor (Table 2).

3.3 Versatility of TA and EFE Cascade

Owing to the success of combining GabT with EFE when converting **1a** into **2a**, the substrate scope of the cascade reaction was further investigated using BCTA [26]. The conversion of keto acids **1a**–**1 g** was conducted using an equivalent amount of L-Glu. Among the products of this study, four (**2a**–**2d**) were unnatural amino acids and the other three (**2e**–**2 g**) were natural hydrophobic amino acids.

As shown in Table 3, the results of the BCTA and EFE cascade showed that keto acid conversion was substantially enhanced to over 99%, with perfect enantioselectivity (> 99%), in each case. However, BCTA and EFE showed different conversions when the reaction was catalyzed by BCTA alone (Table 3). The conversion of TA and the EFE cascade proved that decomposing byproduct α-KG using EFE was an excellent method for making transamination irreversible. Furthermore, the decomposition of α-KG might also reduce byproduct inhibition of TAs (Fig. S5). This TA and EFE cascade shows versatility and applications of TA-catalyzed asymmetric synthesis using only equivalent amount of L-Glu as amino donor are currently being investigated.

Table 2 Equilibrium shifting effect of coupling EFE with TA

Enzymes	TA[a]	TA[b]	TA & EFE[b]
Conversion of **1a** (%)	55.6 ± 0.6	56.0 ± 0.4	> 99[c]
Yield of **2a** (%)	54.5 ± 0.8	54.9 ± 1.2	98.2 ± 0.9
α-KG concentration (mM)	5.5 ± 0.2	5.3 ± 0.3	N.D.[d]

[a]Reaction mixtures contained L-Glu (10 mM), **1a** (10 mM), and PLP (0.2 mM) in sodium phosphate buffer (50 mM, pH 7.4). Purified GabT (1.28 g/L) was added to the reaction. The reaction mixtures were analyzed by HPLC after reacting for 6 h

[b]Reaction mixtures contained L-Glu (10 mM), **1a** (10 mM, Scheme 2), PLP (0.2 mM), L-Arg (3.5 mM), and Fe^{2+} (0.5 mM) in sodium phosphate buffer (50 mM, pH 7.4). Purified GabT (1.28 g/L) with or without purified EFE (10.6 g/L) were added into the reaction. The reaction mixtures were analyzed by HPLC after reacting for 6 h

[c]No obvious **1a** was detected

[d]No obvious α-KG was detected

Table 3 Versatility of equilibrium shifting effect of TA and EFE cascade

Entry	Substrate	Conversion of **1** (%) by TA[a]	Conversion of **1** (%) by TA & EFE[b]	*ee* of **2** (%) by TA & EFE
1	**1a**	55.8 ± 0.8	> 99	> 99
2	**1b**	58.3 ± 0.8	> 99	> 99
3	**1c**	55.2 ± 0.3	> 99	> 99
4	**1d**	44.6 ± 1.2	> 99	> 99
5	**1e**	42.3 ± 1.7	> 99	> 99
6	**1f**	50.0 ± 1.7	> 99	> 99
7	**1 g**	45.4 ± 1.4	> 99	> 99

[a]Reaction mixtures contained L-Glu (10 mM), keto acids (10 mM, **1a**–**1 g**), and PLP (0.2 mM) in sodium phosphate buffer (50 mM, pH 7.4). Purified BCTA (3.6 g/L) was added into the reaction. Conversion and *ee* were analyzed by HPLC after reacting for 6 h

[b]Reaction mixtures contained L-Glu (10 mM), keto acids (10 mM, **1a**–**1 g**), PLP (0.2 mM), L-Arg (3.5 mM), and Fe^{2+} (0.5 mM) in sodium phosphate buffer (50 mM, pH 7.4). Purified BCTA (3.6 g/L) and purified EFE (10.6 g/L) were added into the reaction. Conversion and *ee* were analyzed by HPLC after reacting for 6 h

4 Conclusions

In summary, a novel strategy to make the transamination reaction in the asymmetric synthesis of optically pure amino acids irreversible was developed. Compared with reported procedures, this approach using EFE has the major advantage that the keto acid amino acceptor can be nearly completely converted using only one equivalent of amino donor, L-Glu.

Furthermore, this strategy might also be used in the synthesis of D-amino acids catalyzed by D-amino acid TA and using D-Glu from racemic Glu as the amino donor. This strategy shows great potential for the production of chiral amino acids using Glu as an amino donor.

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Compliance with Ethical Standards

Conflict of interest The authors declare no commercial or financial conflict of interest.

References

1. Kelly SA, Pohle S, Wharry S, Mix S, Allen CCR, Moody TS, Gilmore BF (2017) Chem Rev 118:349–367
2. Koszelewski D, Tauber K, Faber K, Kroutil W (2010) Trends Biotechnol 28:324–332
3. Taylor PP, Pantaleone DP, Senkpeil RF, Fotheringham IG (1998) Trends Biotechnol 16:412–418
4. Mathew S, Yun H (2012) ACS Catal 2:993–1001
5. Guo F, Berglund P (2017) Green Chem 19:333–360
6. Slabu I, Galman JL, Lloyd RC, Turner NJ (2017) ACS Catal 7:8263–8284
7. Meier RJ, Gundersen MT, Woodley JM, Schürmann M (2015) ChemCatChem 7:2594–2597
8. Höhne M, Bornscheuer UT (2012) In: Drauz K, Gröger H, May O (eds) Enzyme catalysis in organic synthesis. Wiley, Weinheim
9. Park E-S, Dong J-Y, Shin J-S (2013) ChemCatChem 5:3538–3542
10. Abu R, Woodley JM (2015) ChemCatChem 7:3094–3105
11. Schulz A, Tripier PT,D, Bartsch K (1990) Appl Environ Microbiol 56:1–6
12. Cho B-K, Seo J-H, Kang T-W, Kim B-G (2003) Biotechnol Bioeng 83:226–234
13. Hanzawa S, Oe S, Tokuhisa K, Kawano K, Kobayashi T, Kudo T, Kakidani H (2001) Biotechnol Lett 23:589–591
14. Klaus Bartsch RS, Schulz A (1996) Appl Environ Microbiol 62:3794–3799
15. Li T, Kootstra AB, Fotheringham IG (2002) Org Process Res Dev 6:533–538
16. Hong E, Cha M, Yun H, Kim B-G (2010) J Mol Catal B Enzym 66:228–233
17. Xian M, Alaux S, Sagot E, Gefflaut T (2007) J Org Chem 72:7560–7566
18. Schrittwieser JH, Velikogne S, Hall M, Kroutil W (2018) Chem Rev 118:270–348
19. Nagahama K, Ogawa T, Fujii T, Tazaki M, Tanase S, Morino Y, Fukuda H (1991) J Gen Microbiol 137:2281–2286
20. Fukuda H, Ogawa T, Ishihara K, Fujii T, Nagahama K, Omata T, Inoue Y, Tanase S, Morino Y (1992) Biochem Biophy Res Commun 188:826–832
21. Eckert C, Xu W, Xiong W, Lynch S, Ungerer J, Tao L, Gill R, Maness PC, Yu J (2014) Biotechnol Biofuels 7:33
22. Yin X, Wu J, Yang L (2018) Appl Microbiol Biotechnol 102:4425–4433
23. Meiji Seika Kaisha LTD (2010) US Patent 0,063,313
24. Pesez M (1959) J Pharm Pharmacol 11:475–476
25. Zeiss HJ (1994) Pestic Sci 41:269–277
26. Yu X, Wang X, Engel PC (2014) FEBS J 281:391–400
27. Nagahama K, Ogawa T, Fujii T, Tazaki M, Goto M, Fukuda H (1991) J Gen Microbiol 137:1641–1646
28. Martinez S, Hausinger RP (2016) Biochemistry 55:5989–5999
29. Martinez S, Fellner M, Herr CQ, Ritchie A, Hu J, Hausinger RP (2017) J Am Chem Soc 139:11980–11988
30. Zhang Z, Smart TJ, Choi H, Hardy F, Lohans CT, Abboud MI, Richardson MSW, Paton RS, McDonough MA, Schofield CJ (2017) Proc Natl Acad Sci 114:4667–4672

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