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Graphical Abstract

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Anwei Hou[a], Zixin Deng[a, b], Hongmin Ma[a, *] and Tiangang Liu[a, b, **]
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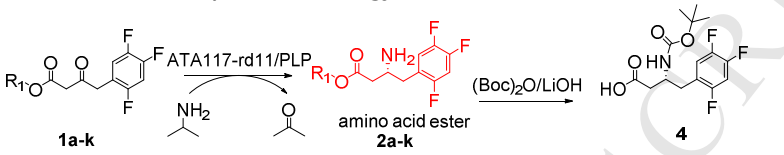

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ABSTRACT

We reported herein a new enzymatic route to synthesize sitagliptin intermediate using an aminotransferase. Substrate profile indicated that hydroxyethyl-3-oxo-4-(2,4,5-trifluorophenyl)butanoate, among 11 analogs, showed the best biocatalytic performance, partially due to its best solubility in the enzymatic system. The corresponding amino esters showing strong product inhibition on the reaction, were inclined to autohydrolyze, thus driving the reaction forward, which indicated the contribution of the rapid hydrolysis of hydroxyethyl ester to the biocatalytic performance. The reaction was performed at 100 mM with 82% conversion in 24 h. The amino ester product was further transformed to Boc-(*R*)-3-amino-4-(2,4,5-trifluorophenyl)butyric acid, the key intermediate of sitagliptin.

1. Introduction

Sitagliptin, marketed as a phosphate salt under the trade name Januvia, is an oral anti-diabetic drug of the dipeptidyl peptidase-4 (DPP-4) inhibitor class.[1] It was developed by Merck and was approved by the U.S. Food and Drug Administration (FDA) in 2006. Sitagliptin is the top-selling drug in its class and has brought in about $6 billion in 2014 for Merck.[2]

Boc-(*R*)-3-amino-4-(2,4,5-trifluorophenyl)butyric acid **4**, the key intermediate of sitagliptin, is mainly synthesized through the hydrolysis and protection of 3-amino-4-(2,4,5-trifluorophenyl)butyric ester **2**. So far, many studies have focused on the synthesis of the above-mentioned compound: some have used the induction of the chiral amino group with a chiral pyrazine compound followed by Arndt-Eistert Homologation to obtain **4**,[3,4] while others used the stereoselective reduction of methyl 3-oxo-4-(2,4,5-trifluorophenyl)butanoate to obtain an *S* configuration chiral alcohol, followed by the Mitsunobu reaction to obtain *R*-3-benzyloxyamino-4-(2,4,5-trifluoruophenyl)butyric acid.[5,6] In addition to these methods, asymmetric resolution of *rac*-3-amino-4-(2,4,5-trifluorophenyl)butyric ester with mandelic acid and other organic acids,[7,8] asymmetric hydrogenation of enamines with $[Rh(COD)Cl]_2$,[9] enzymatic resolution of *rac*-N-acetyl-2-amino-3-(2,4,5-trifluorophenyl) propanoic acid with α-chymotrypsin followed by Arndt-Eistert reaction,[10] and asymmetric synthesis of *R*-3-amino-4-(2,4,5-trifluorophenyl) butyric ester with aminotransferase PYR-1 from *Mycobacterium vanbaalenii* have also been adopted in some studies to obtain **4**.[11,12] Recently, enzymatic routes have been attracting increasing attention because of their eco-friendliness, high stereoselectivity, and catalytic efficiency;[13-15] however, so far only a few studies have focused on aminotransferase-based synthesis of **4**.[11,12] In this study, we presented a chem-enzymatic route for the synthesis of this intermediate, as described in Scheme 1. Our method involves the transformation of the ketoester to amino ester by using an aminotransferase, followed by hydrolysis and protection to form **4**.

An aminotransferase variant, ATA117-rd11, from *Arthrobacter* sp. was chosen owing to its broad substrate profile,[16-18] and the fact that it had been specifically developed for the asymmetric catalysis of pro-sitagliptin.[16] The enzymatic binding pocket can be divided into a larger pocket and a smaller one. [16] Considering the ester part of the substrates should be accommodated in the larger one to ensure satisfactory stereoselectivity,[16,19] some large substituents of the ester, such as benzyl, phenylethyl, and cyclohexyl groups, were chosen. Some small substituents such as the methyl, ethyl, and propyl groups were also chosen to fully map the substrate profile of the enzyme.

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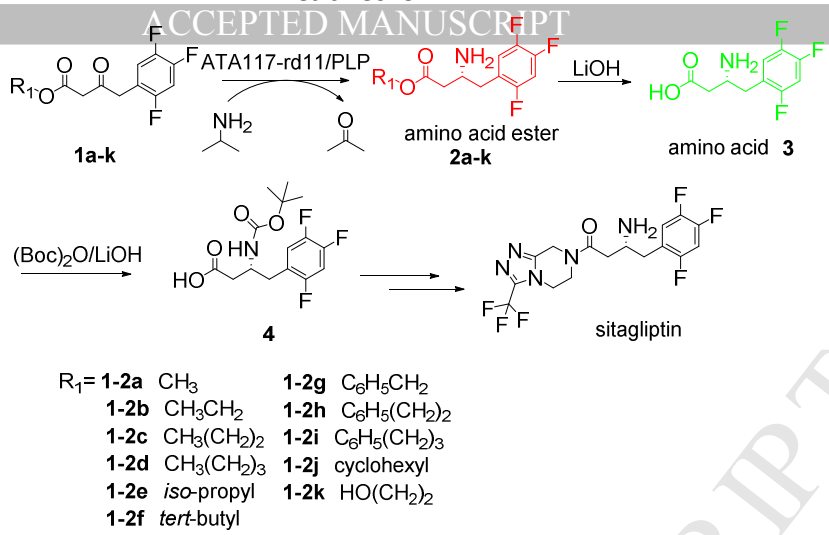

Scheme 1. The chem-enzymatic route for the synthesis of Boc-(*R*)-3-amino-4-(2,4,5-trifluorophenyl)-butyric acid **4**

Further, 1 M isopropylamine was chosen as the amino donor to accelerate the reaction and to increase the conversion.[16]

2. Results and discussion

2.1. Enzymatic reactions using different substrates

The enzymatic activities as well as the biocatalytic performances of the eleven substrates including alkyl esters (methyl **1a**, ethyl **1b**, *n*-propyl **1c**, *n*-butyl **1d**), branched alkyl esters (*iso*-propyl **1e**, *tert*-butyl **1f**), aromatic esters (benzyl **1g**, phenylethyl **1h**, phenylpropyl **1i**), the cyclohexyl ester **1j**, and the hydroxyl ethyl ester **1k** were tested (Table 1, Fig. 1). For the alkyl esters, as the chain prolonged, the enzyme activity increased almost linearly from $(1.70 \pm 0.05)\times10^{-3}$ U/mL to $(14.06 \pm 0.38)\times10^{-3}$ U/mL (Table 1, **1a-d**). With the hydroxyl group, the activity of **1k** became lower than that of **1b** (Table 1, **1k**). The activity of **1e** was almost same as that of **1d**; when the *iso*-propyl group was substituted with the *tert*-butyl group, the activity decreased (Table 1, **1e-f**). **1g** had the highest activity among all the 11 substrates; when the chain prolonged, the activity decreased unlike what was observed for the alkyl esters (Table 1, **1g-i**). It seemed that, among the aromatic esters, **1g** containing the benzyl group was more similar to pro-sitagliptin in structure as they both have the methylene-aromatic ring motif, which probably granted it the highest activity. Despite the highest activity of **1g**, it had poor solubility in the enzymatic system: about 1 mM **1g** was required to ensure that the reaction mixture was homogeneous. However, high substrate concentrations are necessary for the industrial application of enzymatic reactions. In our enzymatic reaction mixture, DMSO was chosen as the cosolvent to increase the solubility of the substrates as described previously.[16] Our attempts to find alternative solvents were unsuccessful: methanol and ethanol were tested, but they caused the transesterification of the substrates in the enzymatic system, and other water-miscible organic solvents such as DMF and THF did not show better behavior than DMSO. Moreover, it was found that, among the 11 substrates, **1k** and **1b** had good solubility in the enzymatic system. The substrates were then tested at a raised concentration of 20 mM (Fig. 1). At this concentration, the reaction mixture of all of the substrates were heterogeneous with **1k** as the only exception, and those of **1a** and **1b** were even a little turbid. The reaction tendencies of the 11 substrates at this higher concentration were totally different from those at low concentrations, and the products formed were found to be prone to undergo autohydrolysis to form 3-amino-4-(2,4,5-trifluorophenyl)butyric acid **3** at high substrate concentrations (Scheme 1, Fig. 1). Therefore, the conversions of all the substrates into the amino esters **2a-k** and the amino acid **3** were analyzed. Surprisingly, **1b** (conversion 41.66%), and **1k** (conversion 37.73%) showed the highest conversion in the first 1 h, while **1g** (conversion 24.98%) showed lower conversion. This phenomenon may be attributed to the poor solubility of **1g** and the good solubility of **1b** and **1k** in the enzymatic system. In the fourth hour, the conversion of **1b** and **1k** was 90.52% and 82.93%, respectively, and that of **1g** was only 45.66%, indicating that at high concentrations, **1b** or **1k** should be a better candidate than **1g**. In the eighth hour, the conversion of **1b** reached 95.87% and that of **1k** was 97.36%, indicating that the conversion of **1b** increased only by 5% during the last four hours compared with the 15% increase in the conversion of **1k**. Upon comparing **1b** and **1k** carefully, it was found that the amino product **2k** was easier to hydrolyze than **2b**, because in 4 and 8 h, **2k** hydrolyzed 33.21% and 58.48%, respectively, while **2b** hydrolyzed 16.59% and 34.48%. This autohydrolysis might reduce product inhibition and thus promote the enzymatic reaction.

Table 1 Enzyme activity of the different substrates

substrates	Enzyme activity[b] U[a]/mg	order
1a	$(1.70\pm0.05)\times10^{-3}$	11
1b	$(5.65\pm0.44)\times10^{-3}$	8
1c	$(9.69\pm0.39)\times10^{-3}$	4
1d	$(14.06\pm0.38)\times10^{-3}$	3
1e	$(14.11\pm0.50)\times10^{-3}$	2
1f	$(9.05\pm0.03)\times10^{-3}$	5
1g	$(18.94\pm0.47)\times10^{-3}$	1
1h	$(6.53\pm0.72)\times10^{-3}$	7
1i	$(6.58\pm0.96)\times10^{-3}$	6
1j	$(2.53\pm0.17)\times10^{-3}$	10
1k	$(2.63\pm0.34)\times10^{-3}$	9

[a] One unit of ATA117-rd11 was defined as 1 mg crude enzyme producing 1 μmol of the amino product per minute.

[b] The values are given as mean ± SD of triplicates.

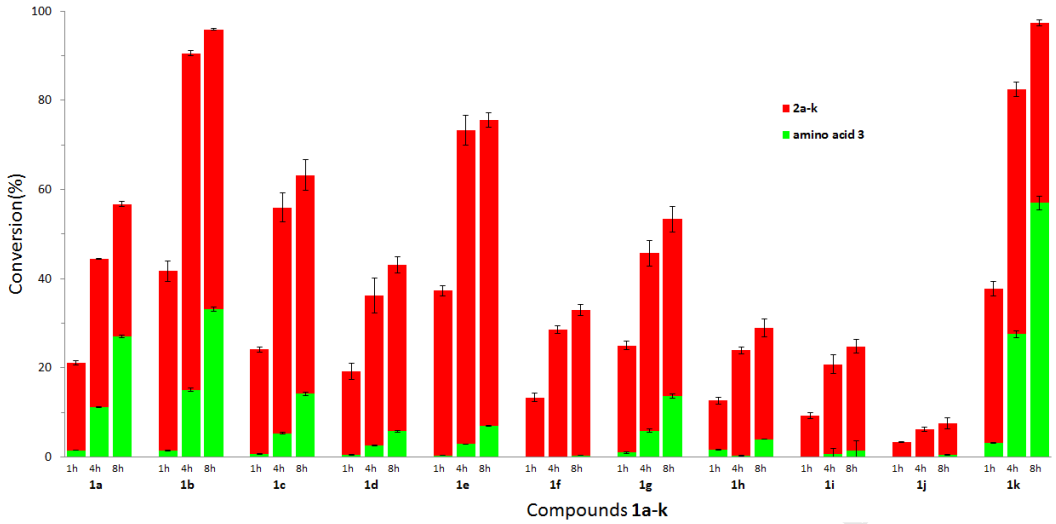

Fig. 1. Enzymatic reactions for different substrates at 1, 4, and 8 h. Red color represents the conversion of the substrates **1a-k** into the corresponding products **2a-k**, and green color represents the conversion of the substrates into the hydrolyzed amino acid **3**. Error bars represent standard deviation of triplicates.

2.2. The inhibition of amino acid 3 and amino ester 2b to the aminotransferase

The hydroxyl substitution of **1k** granted it the highest solubility, which is pivotal for high reaction rate caused by the increased concentration in the aqueous phase. The fact that the amino ester **2k** was easiest to hydrolyze indicated that this behavior might reduce product inhibition on the enzyme. Hence, the enzymatic activities at different concentrations of the amino acid **3** and amino ester **2b** were tested (Fig. 2). Considering the poor solubility of the amino acid **3** in a solution containing DMSO or not at neutral pH, pH 10 was adopted to ensure good solubility of **3** in the reaction system. Experimentally, the activity of the aminotransferase at the presence of **3** at 100 mM was the same as that without the addition of the amino acid, which proved that **3** has no inhibitory effect on the enzyme reaction at concentrations of 0–100 mM. In the case of *rac*-amino ester **2b**, at 20 mM of the *rac*-amino ester **2b**, the enzyme showed only about 60% relative activity, and the relative activity ceased to reduce at higher concentrations. The inhibition of enzyme activity with *rac*-amino ester **2b** is shown in Fig 2. Thus, the amino ester **2b** exerted an obvious inhibitory effect on enzyme activity. The reason why the inhibition caused by **2b** did not increase with increasing concentrations of **2b** may be attributed to the poor solubility of the *rac*-amino acid ester **2b** in the reaction mixture, which was indicated by the fact that the reaction solution became turbid when **2b** was used at a concentration of 40 mM.

2.3. Optimization of the enzymatic reaction conditions

Because of the good solubility and apparent biocatalytic behavior of **1k** and the auto-hydrolysis ability of the corresponding amino ester **2k**, **1k** was believed to be the best substrate for use in industrial applications. The reaction conditions, such as the concentrations of **1k** and DMSO, were then optimized, as shown in Fig. 3. At 50 mM of **1k**, 85% conversion was observed in 16 h and 95% conversion could be achieved in 24 h in the presence of 10% or 20% DMSO. At 100 mM, the conversion of **1k** reduced to 60% in 16 h and to about 80% in 24 h, which may possibly resulte from the slight decomposition during the reaction. The conversion at the presence of 10% DMSO was slightly less than that at 20% DMSO. Thus, 100 mM of **1k** and 20% DMSO were chosen as the optimum conditions for further experiments.

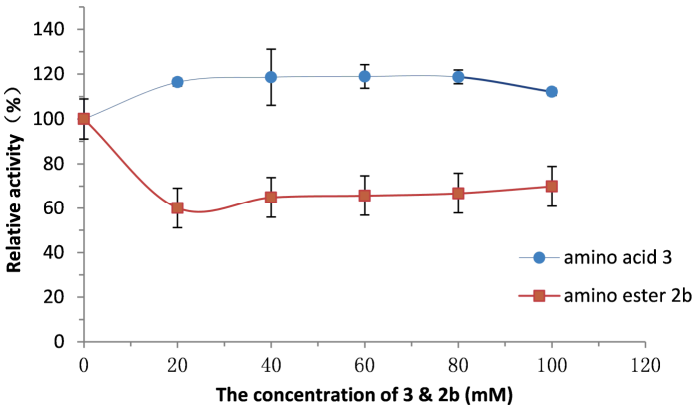

Fig. 2. Inhibition of enzyme activity by the amino ester **2b** and the amino acid **3**. Error bars represent standard deviation of triplicates.

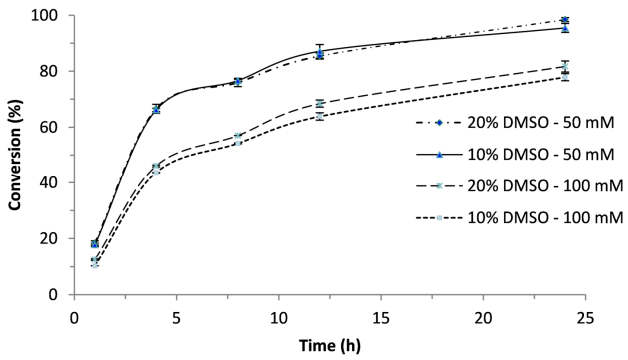

Fig. 3. Time course of **1k** at different concentrations of **1k** and in the presence of 20% or 10% DMSO. Error bars represent standard deviation of triplicates.

2.4. Preparation of Boc-(*R*)-3-amino-4-(2,4,5-trifluoro-phenyl)butyric acid 4

As the amino acid ester **2k** tended to hydrolyze to form the amino acid **3**, which was hardly soluble in organic solvents such

as ethyl acetate, it was difficult to use organic solvents to extract all the products needed. However, Boc-(*R*)-3-amino-4-(2,4,5-trifluorophenyl)butyric acid **4** shows good solubility in organic solvents. Therefore, we developed an optimized method to synthesize **4** after the enzymatic reaction. Because the concentration of isopropyl amine (1 M) was much higher than that of the products, it was necessary to remove as much isopropyl amine as possible to avoid the consumption of Boc anhydride during the chemical synthesis of **4.** The pH of the biocatalytic product was adjusted to 10 to remove isopropyl amine as a volatile free base by rotary evaporation, and this alkaline condition also allowed **2k** to become completely hydrolyzed to form amino acid **3**. Using this strategy, only about 2 eq. Boc anhydride was added and all of the amino acid **3** was transformed to **4**. Then, using a 10 mL scale reaction with 100 mM **1k** in 20% DMSO, about 200 mg of Boc acid **4** was obtained (60.6% yield, 98.6% *ee*) as a white solid.

3. Conclusion

Here we developed a new enzymatic route to synthesize the intermediate of sitagliptin using an aminotransferase. Through substrate screening of 11 substrates, hydroxyethyl-3-oxo-4-(2,4,5-trifluorophenyl)butanoate **1k** was found to be the best substrate for use in industrial applications. **1k** had the best biocatalytic performance owing to its high solubility in the enzymatic system and its corresponding amino ester **2k** was easy to autohydrolyze to drive the reaction forward. The reaction could be performed using 100 mM of **1k** and showed 82% conversion in 24 h. The amino product was then transformed to Boc-(*R*)-3-amino-4-(2,4,5-trifluorophenyl) butyric acid **4**, the key intermediate of sitagliptin, with 60.6% total yield and 98.6% *ee*.

4. Experimental section

4.1. General

2,4,5-Trifluorophenylacetic acid, Meldrum's acid, and ketoester **1a** were purchased from Chemlin Chemical Industry Co. (Nanjing, China). N,N-dimethylaminopyridine, N,N-diisopropyl-ethylamine, and pyridoxal 5'-phosphate (PLP) were purchased from Energy Chemicals (Shanghai, China). The ketoesters **1b-k** were synthesized as described previously.[20] All other organic substrates (AR or AG) were obtained from commercial sources and used without further purification. ^{1}H NMR spectra and ^{13}C NMR were recorded on an Agilent 400 MHz instrument (DirectDrive2)(California, USA). Chemical shifts were recorded in ppm downfield from tetramethylsilane. J values were given in Hz. Abbreviations used in ^{1}H NMR are s (singlet), d (doublet), t (triplet), q (quartet), and m (multiplet). High-resolution mass spectrometry (HRMS) experiments were performed using a Thermo LTQ XL Orbitrap instrument (Massachusetts, USA).

4.2. Expression of ATA117-rd11

The ATA117-rd11 gene was codon-optimized and synthesized using a C-terminal His-tag by Tianyi Huiyan Co. (Wuhan, China). The gene was then inserted into a pRSFDuet-1 expression vector at the BamHI and XhoI restriction sites, and the plasmid was transformed into *Escherichia coli* BL21 (DE3) cells. The recombinant cells were cultivated in 500 mL LB broth containing 50 mg/L kanamycin at 37°C. When the OD600 reached 0.6–0.8, enzyme expression was induced by the addition of *iso*-propyl-β-D-thiogalactoside (IPTG) to obtain a final concentration of 1 mM. The cells were then cultivated for another 16–20 h, after which they were harvested by centrifugation (4500 rpm, 15 min, 4°C) and the supernatant was discarded. Next, the cell pellets were resuspended in 100 mM triethanolamine (chloride) buffer (pH 8.5) containing 1 mM PLP and were then harvested by centrifugation using the same conditions as described above. One gram of the wet cell pellets were then resuspended in 10 mL cold triethanolamine (chloride) buffer containing PLP, and the suspension was subjected to ultrasonic disruption for 12 min. Ultrasonic cell disruption followed by centrifugation (16000 rpm, 20 min, 4°C) finally yielded the crude enzyme solution. The crude enzyme solution could be used immediately or stored at 4°C for several days; alternatively, the cell pellets can also be stored at -80°C until use. Protein concentrations were determined using Bradford method.

4.3. Preparation of *rac* 2a-k compounds

The *rac* compounds were prepared using a previously described method[21] with minor modifications. The ketoester **1b** (198 mg, 0.76 mmol), ammonium acetate (586 mg, 7.6 mmol), and sodium cyanoborohydride (50 mg, 79 mmol) were added in 4 mL methanol, and the solution was stirred overnight at room temperature. Concentrated HCl was then added to the solution until the final pH was 2, and then the methanol was removed by rotary evaporation. Next, 5 mL water was added to the residue and 10 M NaOH aqueous solution was used to adjust the pH to 10. Ethyl acetate (2× 5 mL) was added to this solution to extract the product. The organic layers were combined and dried using anhydrous sodium sulfate and concentrated to obtain the crude product. The crude product was then purified by preparative thin layer chromatography (PE/EA=5/3) to yield compound **2b** (86 mg, 43.9%) as a colorless oil. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.04 (ddd, *J* = 10.5, 8.7, 6.7 Hz, 1H), 6.90 (ddd, *J* = 10.1, 9.2, 6.6 Hz, 1H), 4.13 (q, *J* = 7.1 Hz, 2H), 3.52 – 3.34 (m, 1H), 2.78 – 2.56 (m, 2H), 2.50 – 2.23 (m, 2H), 1.25 (t, *J* = 7.1 Hz, 3H). ^{13}C NMR (101 MHz, Chloroform-d) δ 172.00, 156.10 (ddd, J = 244.1, 9.2, 2.6 Hz), 148.74 (ddd, J = 250.0, 14.3, 12.4 Hz), 146.56 (ddd, J = 244.8, 12.4, 3.6 Hz), 121.84 (ddd, J = 18.3, 5.5, 4.2 Hz), 118.95 (ddd, J = 18.9, 6.1, 1.4 Hz), 105.48 (dd, J = 28.7, 20.8 Hz), 60.58, 48.57, 41.70, 36.37, 14.17. HRMS (ESI) calculated for $C_{12}H_{15}F_3NO_2$ $[M+H]^+$: *m/z* 262.1049; *m/z* found: 262.1051.The other *rac* compounds were prepared in a similar way.

4.4. Enzyme activity with different substrates

60 μL crude enzyme solution at 0.5-1 mg/mL concentration, 100 μL of 60 mM isopropyl amine (chloride) solution (pH 8.5), and 40 μL of a solution of **1a-k** (5 mM) in DMSO were added into a 2 mL Eppendorf tube. The mixture was then placed in a shaking incubator at 45°C for 0.5 h, during which the conversions were guaranteed to be less than 10 %. Then 0.8 mL acetonitrile was then added to the reaction mixture. The conversion was then analyzed by HPLC.

4.5. Enzymatic reactions using different substrates

A 125 μL crude enzyme solution, 275 μL of 1.83 M isopropyl amine (chloride) solution (pH 8.5), and 100 μL of a solution of **1a-k** (100 mM) in DMSO were added into a 2 mL Eppendorf tube. The mixture was then placed in a shaking incubator at 45°C for 8 h. Twenty microliters of the sample was taken at 1, 4, and 8

h and diluted with 380 μL methanol for use in HPCL to analyze conversation.

4.6. The inhibition of amino acid 3 to the aminotransferase

A 50 μL crude enzyme solution, 110 μL of 1.83 M isopropyl amine (chloride) solution (pH 10) containing 0–183 mM amino acid **3**, and 40 μL of a solution of **1b** (100mM) in DMSO were added into a 2 mL Eppendorf tube. The mixture was then placed in a shaking incubator at 45°C for 30 min. Twenty microliters of the sample was taken and diluted with 380 μL methanol, and the conversation was analyzed using HPLC. The relative activity was determined by analysis of the consumption of the substrate **1b** at the presence of amino acid **3,** and the activity at the absence of amino acid **3** was used as the control.

4.7. The inhibition of amino ester 2b to the aminotransferase

A 50 μL crude enzyme solution, 110 μL of 1.83 M isopropyl amine (chloride) solution (pH 8.5), 20 μL of a solution of **1b** (200 mM) in DMSO, and 20 μL of a solution of **2b** (0–1000 mM) in DMSO were added into a 2 mL Eppendorf tube. The mixture was then placed in a shaking incubator at 45°C for 30 min. Twenty microliters of the sample was taken and diluted with 380 μL methanol, and the conversation was analyzed by HPLC. The relative activity was determined by analysis of the consumption of the substrate **1b** at the presence of amino ester **2b**, and the activity at the absence of amino ester **2b** was used as the control.

4.8. Enzymatic reaction using different concentrations of 1k

A 125 μL crude enzyme solution, 275 μL of 1.83 M isopropyl amine (chloride) solution (pH 8.5), and 100 μL of a solution of **1k** (100–500 mM) in DMSO were added into a 2 mL Eppendorf tube. The mixture was then placed in a shaking incubator at 45°C for 24 h. Twenty microliters of the sample was taken at 1, 4, 8, 12, and 24 h, and diluted with 380 μL methanol to analyze the conversation using HPLC.

4.9. Preparation of Boc-(*R*)-3-amino-4-(2,4,5-trifluoro-phenyl)butyric acid 4

A 2.5 mL crude enzyme solution, 5.5 mL of 1.83 M isopropyl amine (chloride), and a 2 mL solution of **1k** (500 mM, 1 mmol) in DMSO were added into a 50 mL sample bottle. The bottle was then incubated at 45°C for 24 h, and the conversion was found to be 80%. Anhydrous lithium hydroxide (192 mg, 8 mmol) was added to this mixture and incubated for another 3 h to hydrolyze all the products. The mixture was then centrifuged (6000 rpm, 10 min), and the residue was washed twice with 4 mL 0.4 M lithium hydroxide aqueous solution. Next, the aqueous layer was mixed and concentrated in high vacuum to remove the remaining isopropyl amine. When almost all of the isopropyl amine was removed, 3 mL tetrahydrofuran and di-tert-butyl pyrocarbonate (427 mg, 1.96 mmol) was added to this solution. The mixture was then stirred overnight at 35°C to complete the reaction. Ethyl acetate (2× 15 mL) was used to extract the product. The organic layer was combined and dried using anhydrous sodium sulfate, and then concentrated by rotary evaporation. The crude product was purified by silica column chromatography (PE/EA/acetic acid=100/50/1) to yield Boc acid **4** (200 mg, 60.6% yield, 98.6% *ee*) as a white solid. ^{1}H-NMR (400 MHz, Methanol-d4) δ 7.23 – 7.13 (m, 1H), 7.17 – 7.01 (m, 1H), 4.92 (s, 2H), 4.19 – 4.07 (m, 1H), 2.92 (dd, J = 13.8, 5.0 Hz, 1H), 2.65 (ddd, J = 13.8, 9.2, 1.4 Hz, 1H), 2.63 – 2.41 (m, 2H), 1.32 (s, 9H). ^{13}C NMR (101 MHz, Chloroform-d) δ 176.08, 174.84, 157.12, 155.12, 150.14, 147.79, 145.30, 121.04, 118.89, 105.36 (dd, J = 28.6, 20.8 Hz), 79.90, 47.56, 37.80, 32.97, 28.21. HRMS (ESI) calculated for $C_{15}H_{17}F_3NO_4$ [M-H]$^-$: *m/z* 332.1115; *m/z* found: 332.1123.

4.10. Analytical methods

The conversion rate was determined using an ultimate 3000 HPLC equipped with a Waters Xterra RP C18 column (3.9 × 150 mm, 5 μm) using 10 mM NH_4Ac/acetonitrile as the eluent. The gradient was 1–10 min: 10%-90% acetonitrile, 10–11 min: 90% acetonitrile, 11–12 min: 90%-10% acetonitrile, and 12–15 min 10% acetonitrile.

The enantiomeric excess of Boc-(*R*)-3-amino-4-(2,4,5-tri-fluorophenyl)butyric acid **4** was determined using a Shimadzu LC 20 with CHIRALPAK AD-H (4.6 × 250 mm, 5 μm) using Hexane/ IPA = 90/10 as the eluent at a flow rate of 1 mL/min.

Legends

Scheme 1 The chem-enzymatic route for the synthesis of Boc-(*R*)-3-amino-4-(2,4,5-trifluorophenyl)-butyric acid **4**.

Table 1. Enzyme activity of the different substrates.

Fig. 1. Enzymatic reactions for different substrates in 1, 4, and 8 h. Red color represents the conversion of the substrates **1a-k** into the corresponding products **2a-k**, and green color represents the conversion of the substrates into the hydrolyzed amino acid **3**. Error bars represent standard deviation of triplicates.

Fig. 2. Inhibition of enzyme activity by the amino ester **2b** and the amino acid **3**. Error bars represent standard deviation of triplicates.

Fig. 3. Time course of **1k** at different concentrations of **1k** and in the presence of 20% or 10% DMSO. Error bars represent standard deviation of triplicates.

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