Acta Pharmaceutica Sinica B 2017;7(4):510–516

Chinese Pharmaceutical Association
Institute of Materia Medica, Chinese Academy of Medical Sciences

Acta Pharmaceutica Sinica B

www.elsevier.com/locate/apsb
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ORIGINAL ARTICLE

# Steroids hydroxylation catalyzed by the monooxygenase mutant *139-3* from *Bacillus megaterium* BM3

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Received 17 February; received in revised form 16 March 2017; accepted 20 March 2017

**KEY WORDS**

Cytochrome P450;
BM3;
139-3;
Steroids hydroxylation;
1$\alpha$-OH-androstenedione;
Biocatalyst

**Abstract** The search of new substrates with pharmaceutical and industrial potential for biocatalysts including cytochrome P450 enzymes is always challenging. Cytochrome P450 BM3 mutant *139-3*, a versatile biocatalyst, exhibited hydroxylation activities towards fatty acids and alkanes. However, there were limited reports about its hydroxylation activity towards steroids. Herein, an *Escherichia coli*–based whole-cell extract containing the recombinant 139-3 protein was used as the biocatalyst to screen 13 steroids. Results revealed that 139-3 was able to specifically hydroxylate androstenedione (**1**) at 1$\alpha$-position, generating a hydroxylated steroid 1$\alpha$-OH-androstenedione (**1a**). To investigate whether C-1$\alpha$ hydroxylation catalyzed by BM3 mutant *139-3* could be industrially used, an optimization of catalyzing conditions was performed. Accordingly, the BM3 mutant 139-3 enzyme was observed to display maximum activity at 37 °C, under pH 7.0 for 4 h, with 37% transformation rate. Moreover, four *139-3* variants were generated by random mutagenesis with the aim of improving its activity and expanding substrate scope. Surprisingly, these mutants, sharing a common mutated site R379S, lost their activities towards androstenedione (**1**). These data clearly indicated that arginine residue located at site 379 played key role in the hydroxylation activities of 139-3. Overall, these new findings broadened the substrate scope of 139-3 enzyme, thereby expanding its potential applications as a biocatalyst on steroids hydroxylation in pharmaceutical industry.



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Peer review under responsibility of Institute of Materia Medica, Chinese Academy of Medical Sciences and Chinese Pharmaceutical Association.

http://dx.doi.org/10.1016/j.apsb.2017.04.006

## 1. Introduction

Cytochrome P450 BM3 (CYP102A1) from *Bacillus megaterium* is a naturally-occurring $C_{12}$–$C_{20}$ fatty acid hydroxylase and considered as one of the most active monooxygenase so far identified[1,2]. BM3 is a soluble and stable fusion between a catalytic domain involved in substrate oxidation and a diflavin reductase domain responsible for electron transport, thereby making the electron transfer very efficient. These fine properties, together with the ease of over-expression in *Escherichia coli* (*E. coli*) made BM3 a promising candidate for the biocatalysis[3–5]. Therefore, BM3 was studied extensively and many BM3 mutants with broader substrate range and altered region- and stereo-selectivities were generated by laboratory evolution[6–8]. A BM3 variant *139-3* was thus generated by five generations of random mutagenesis[8]. Besides fatty acids, 139-3 was also highly active in hydroxylation of alkanes[8,9] and epoxidation of alkenes[9,10] and steroids[11]. These evidences collectively implicated that 139-3 had a potential to be a versatile biocatalyst. The search for wide-ranging substrates for 139-3 will thus be pharmaceutical or industrial interest.

Steroids are pharmaceutically important compounds[12,13]. The hydroxylations of steroids were deemed to offer access to otherwise inaccessible sites of the steroid compounds or to provide the steroid molecules with diverse modifications for pharmaceutical applications[14,15]. However, the reports of steroids hydroxylation catalyzed by 139-3 were limited, which hindered the extensive applications of 139-3 with therapeutic and industrial interest.

Herein, a compound library containing 13 steroids was used as the substrate to test the hydroxylation activity of 139-3. Results revealed that 139-3 could hydroxylate androstenedione (**1**) at 1$\alpha$-position. Moreover, to investigate weather C-1$\alpha$ hydroxylation catalyzed by BM3 mutant *139-3* could be industrially used, an optimization of catalyzing conditions was performed in this study. To improve the hydroxylation activity and substrates specificity of 139-3, random mutagenesis of 139-3 was performed by error-prone PCR (EP-PCR). Unexpectedly, these resulting mutants lost their 1$\alpha$-hydroxylated activity towards androstenedione (**1**) completely, thereby determining arginine residue at site 379 as one of the key amino acid regulating the 1$\alpha$-hydroxylated activity of 139-3. Undoubtedly, these findings broadened the substrate range of BM3, and thus expanded its huge potential for synthetic biology applications.

## 2. Materials and methods

### 2.1. Steroidal substrates

A total of 13 steroids dissolved in dimethylsulfoxide (DMSO) or dimethylformamide (DMF) were provided as the substrates. The sources of these steroidal substrates were the same as described recently[16]. The detailed structures of these steroidal substrates were listed in Fig. 1.

### 2.2. Heterologous expression of BM3 mutant 139-3 in E. coli

P450 BM3 variant *139-3* was synthesized according to the sequence provided by Glieder et al.[8]. The synthetical sequence was amplified using gene-specific primers (Table S1 in Supplementary information) and the resultant PCR product was then ligated into *Eco*RI/ *Hin*dIII linearized pET-28a (+) (Novagen, Madison, USA) using Seamless Assembly Cloning Kit (CloneSmarter Technologies Inc, Houston, TX, USA). The resulting recombinant plasmid pET28a139-3 was transformed into *E. coli Transetta* (DE3) (TransGen Biotech Co., Ltd., Beijing, China) for heterologous expression. The detailed procedure was performed as described previously[17]. P450 BM3 139-3 was induced to express at 20 °C for 16 h by the addition of isopropyl-$\beta$-d-thiogalactopyranoside (IPTG) with a final concentration of 0.1 mmol/L. The induced cultures were collected by centrifugation at 12,000 × *g* for 5 min and the resulting *E. coli* cells were resuspended in sodium phosphate buffer (0.2 mol/L, pH 7.0) and then lysed with a high pressure homogenizer (800 bar, 3 passes). The resulting supernatant was either checked for protein solubility using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analyses or used as the crude enzyme for measurement of hydroxylation activity.

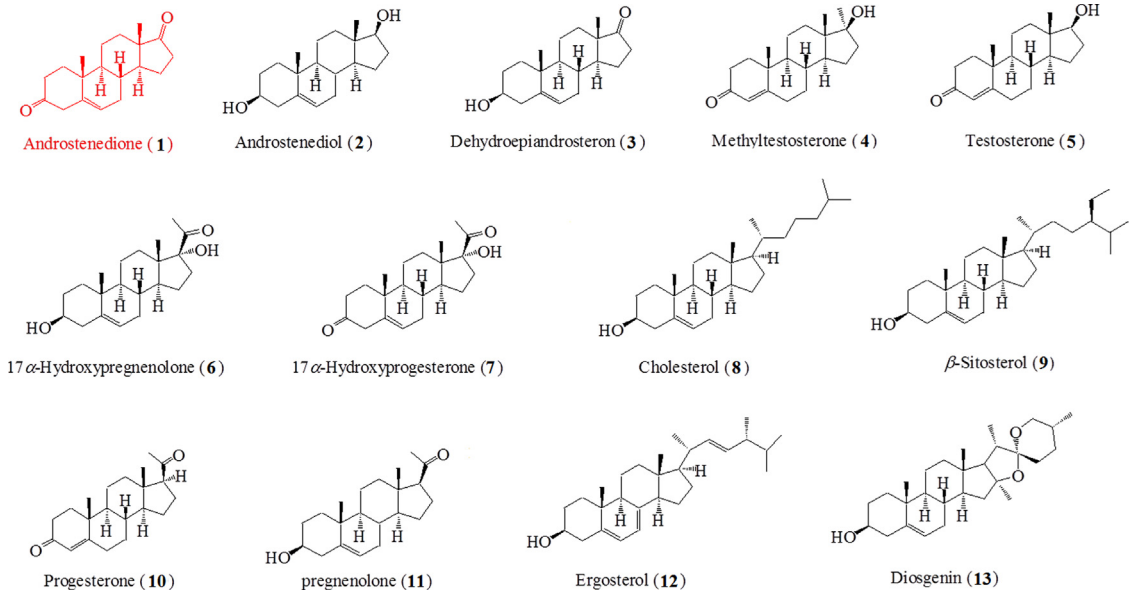


**Figure 1** The structures of the steroidal substrates.

### 2.3. Spectral analysis

To further verify the successful expression of BM3 protein in *E. coli*, a reduced carbon-monoxide (CO) difference spectroscopy analysis based on the whole cell was conducted as described previously[18]. Concretely speaking, 2.4 mL crude enzymes (derived from 10 mL culture) were treated with reducing agent sodium dithionite and then divided equally into two cuvettes. CO was bubbled through one sample cuvette for 1 min, but not the other. After standing for three minutes, the reduced CO difference spectra of the two cuvettes were recorded in the absorbance range from 400 to 500 nm in SpectraMax 190 Microplate Reader (Molecular Devices LLC., CA, USA), respectively. The corresponding difference value between the two cuvettes was thus defined as the reduced CO difference value of the crude enzyme containing 139-3 protein. The crude extract expressing the empty vector alone was used as the control and its reduced CO difference spectrum was also calculated using the same procedure.

### 2.4. Steroids hydroxylation with the recombinant BM3 139-3

The crude enzyme of the recombinant BM3 139-3 was used as the catalyst for the conversion of steroids to their corresponding hydroxyl products. Specifically, a total of 200 μL reaction mixture containing 100 μL sodium phosphate buffer (0.2 mol/L, pH 7.0), 94 μL crude enzyme (derived from 6.6 mL cultures), 2 μL NADPH regenerating system (11 mmol/L $NADP^+$, 33 mmol/L $MgCl_2$ and 33 mmol/L glucose 6-phosphate), 1 μL of 50 mmol/L steroidal substrate, 1 μL of 50 mmol/L NADPH and 2 μL of 40 unit/mL glucose-6-phosphate dehydrogenase were incubated at 37 °C for 4 h. The reaction solution was extracted three times with equal volume of chloroform. The mixed organic extract was evaporated to dryness and reconstituted with 200 μL acetonitrile for HPLC and HPLC–MS analysis.

HPLC procedure was performed on the Thermo Scientific Dionex Ultimate 3000 HPLC system (Thermo Scientific Dionex, USA) with the DAD detector set at the range of 210–284 nm, fitted with a reversed-phase C18 column [YMC-Pack ODS-A (5 μm, 12 nm, 250 mm × 4.6 mm)]. The chromatographic condition was the same as described previously[19]. LC–MS was conducted as reported in our previous publications[19–23].

Moreover, this metabolite was produced on a preparative scale of 30 mL reaction volume containing 500 μmol/L androstenedione (**1**) and the biocatalyst BM3 1393 from 2 L fermentation broth. The resulting product was then collected and its structure was determined by NMR and NOESY analysis as described previously[21].

### 2.5. Optimization of androstenedione hydroxylation by P450 BM3 139-3

A variety of buffers, different temperatures and diverse incubation time were used to optimize reaction conditions of androstenedione (**1**) hydroxylation by P450 BM3 139-3. For measurement of the optimal temperature, reactions were performed in $Na_2HPO_4$/$NaH_2PO_4$ buffer (0.2 mol/L, pH 7.0) at varied temperatures of 4, 20, 30, 37, and 50 °C for 4 h.

To determine pH profiles of androstenedione (**1**) hydroxylation reactions, assays were performed at $Na_2HPO_4$/$NaH_2PO_4$ buffer (0.2 mol/L) with varied pH ranging from 5.5 to 10.0 at a constant temperature of 37 °C for 4 h.

Also, the determination of the optimal incubation time was conducted in this investigation. Specifically, the hydroxylated reactions were performed in $Na_2HPO_4$/$NaH_2PO_4$ buffer (0.2 mol/L, pH 7.0) and 37 °C for varied reaction time of 0.5, 1, 2, 4, 6 and 12 h. The formation of hydroxysteroid in various buffers, temperatures and incubation time were monitored by HPLC. Controls without enzymes were included and three independent biological replicates were performed.

### 2.6. Random mutagenesis of 139-3 gene by error-prone PCR

The P450 BM3 mutant *139-3* gene was subjected to random mutagenesis by EP-PCR as described previously[24]. Plasmid pET28a139-3 was used as the mutagenesis template. Random mutagenesis was performed in a reaction mixture containing $MnCl_2$ (0.1 mmol/L), *Easy Taq* DNA polymerase (TransGen Biotech Co., Ltd., Beijing, China) and primers PCWF and PCWR (Supplementary Table S1). The PCR products were then inserted into pCWori[25] cleaved with *Nde*I and *Bam*HI to generate recombinant plasmids. After sequencing confirmation, the resulting pCWori-derived plasmids were transformed into *E. coli Transetta* (DE3) for heterologous expression. The presence of recombinant proteins was determined by SDS-PAGE analysis. Hydroxylation activity of these mutants was tested by measuring the formation of hydroxylated derivatives from 13 steroid substrates by *in vitro* reactions.

## 3. Results and discussion

### 3.1. Prokaryotic expression of BM3 139-3

The entire open reading frame (ORF) of 139-3 was 3150 bp encoding a polypeptide of 1049 aa with a theoretical molecular weight of 117.75 kDa[8]. The heterologous expression of pET28a139-3 was induced by IPTG in *E. coli* strain *Transetta* (DE3) at 20 °C for 16 h. The specific protein with approximately 118 kDa (Fig. 2) was observed by SDS-PAGE detection, validating the soluble expression of 139-3 protein in bacteria.

### 3.2. Analysis of CO difference spectra of the recombinant BM3 139-3

The reduced CO difference spectrum was usually used to verify the presence of active, recombinant cytochrome P450[18]. In this contribution, the cell-free extract containing 139-3 protein displayed a single broad maximum peak in the reduced CO difference spectrum at 450 nm, which was a characteristic 450-nm absorbance maximum of all cytochrome P450 (Fig. 3). On the contrary, the CO difference spectrum of the control was not detectable in the tested absorbance range (Fig. 3). These data collectively indicated that BM3 139-3 gene had successfully expressed in *E. coli*. Accordingly, the crude enzyme harboring the recombinant 139-3 protein was used as the biocatalyst to react with diverse steroids for the determination of its steroid hydroxylation capacity.

### 3.3. Assay of hydroxylation activity of BM3 139-3 toward steroids

The 139-3 protein was reported to exhibit hydroxylation activity towards fatty acids and alkanes[8], providing a hint that 139-3 might

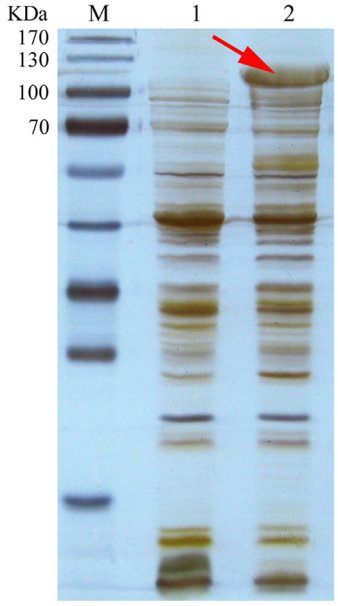


**Figure 2** SDS-PAGE analysis of the crude extract of the transformant containing pET28a139-3 (lane 2) and the empty vector (lane 1). Lane M shows the proteins marker with the indicated molecular masses. The arrow indicated the recombinant 139-3 protein.

have a potential capacity of hydroxylating steroids. The hydroxylation activity of BM3 mutant *139-3* was thus tested towards 13 steroids (Fig. 1) in the present research. When the recombinant BM3 139-3 was incubated with androstenedione (**1**), a new peak with a retention time ($t_r$) of 13 min was detected in HPLC profile (Fig. 4). The retention time of this new product was consistent with that of authentic 1$\alpha$-OH-androstenedione as described previously[16]. On the other hand, when androstenedione (**1**) was added to the control reaction solution containing the empty vector, no any new peak was present in the HPLC profile (Fig. 4). These evidences revealed that the new metabolite with a $t_r$ of 13 min was the enzymatic product of BM3 139-3. Moreover, the ultraviolet spectrum of this new metabolite was similar

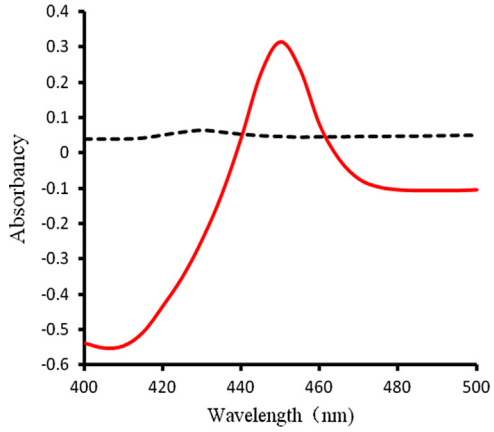


**Figure 3** Reduced CO difference spectra of crude extracts harboring the recombinant 139-3 protein (red line) and the empty vector alone (dashed line).

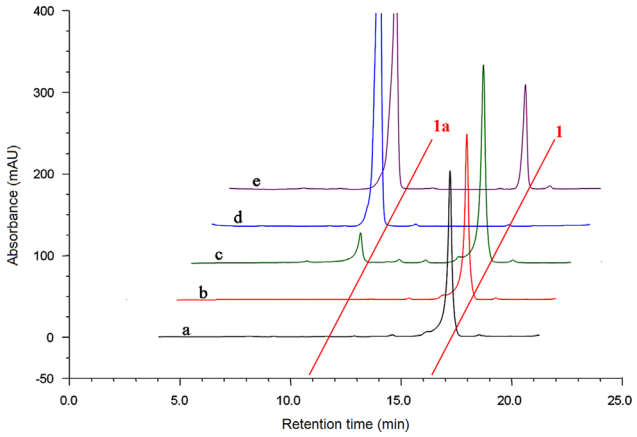


**Figure 4** HPLC chromatograms of androstenedione standard (a); hydroxylated product of androstenedione catalyzed by a whole-cell biocatalyst expressing the empty vector (b) and 139-3 (c); 1$\alpha$-OH-androstenedione standard (d); and the co-injection of reaction mixture containing 139-3 protein and the reference standard 1$\alpha$-OH-androstenedione (e) Compounds. **1** and **1a** show androstenedione and 1$\alpha$-OH-androstenedione standards, respectively.

to that of androstenedione (**1**), suggesting this new metabolite was a derivative of androstenedione (**1**). For further characterization the new metabolite, LC–MS was performed toward the reaction mixture. The newly formed metabolite was thus identified to have a $[M + H]^+$ peak at a $m/z$ value of 303.19342, consistent with that of the authentical 1$\alpha$-OH-androstenedione (Fig. 5). Furthermore, the reaction mixture was co-injected with a reference standard 1$\alpha$-OH-androstenedione standard under the HPLC-based assay. Only a 1$\alpha$-OH-androstenedione peak with no shouldering was found by HPLC analysis (Fig. 4), thereby suggesting that the newly formed reaction product catalyzed by BM3 mutant *139-3* might be 1$\alpha$-OH-androstenedione. To further verify that the newly formed metabolite representing exactly 1$\alpha$-OH-androstenedione, 1.58 mg compound was collected and subjected to NMR (Table 1) and NOESY analysis. As shown in Table 1, this metabolite with $t_r$ of 13 min was assigned exactly as 1$\alpha$-OH-androstenedione.

Since the hydroxylation at 1$\alpha$-position is of special interest for the pharmaceutical industry, more steroids were used as the substrates to investigate the 1$\alpha$-hydroxylated activity of 139-3. However, the BM3 mutant *139-3* had no hydroxylation activity on other 12 steroids including androstenediol (**2**), dehydroepiandrosteron (**3**), methylesterone (**4**), testosterone (**5**), 17$\alpha$-hydroxypregnenolone (**6**), 17$\alpha$-hydroxyprogesterone (**7**), cholesterol (**8**), $\beta$-sitosterol (**9**), progesterone (**10**), pregnenolone (**11**), ergosterol (**12**) and diosgenin (**13**) (Supplementary Figs. S1–12), thus suggesting 139-3 was a highly selective 1$\alpha$-hydroxylase for androstenedione (**1**). Androstenedione (**1**) was the most common precursor of male and female sex hormones. The hydroxylated modifications of androstenedione (**1**), including hydroxylation at positions 1$\alpha$, 7$\alpha$, 9$\alpha$, 11$\alpha$, 14$\alpha$, 15$\alpha$, 16$\alpha$, 1$\beta$, 6$\beta$, 15$\beta$ and 17$\beta$ had been demonstrated previously[16,26]. There were four P450 enzymes displaying 1$\alpha$-hydroxylated activity towards androstenedione (**1**)[16,26]. Compared to these P450 enzymes with 1$\alpha$-hydroxylated activity, 139-3 could exclusively hydroxylate androstenedione (**1**) at 1$\alpha$-position.

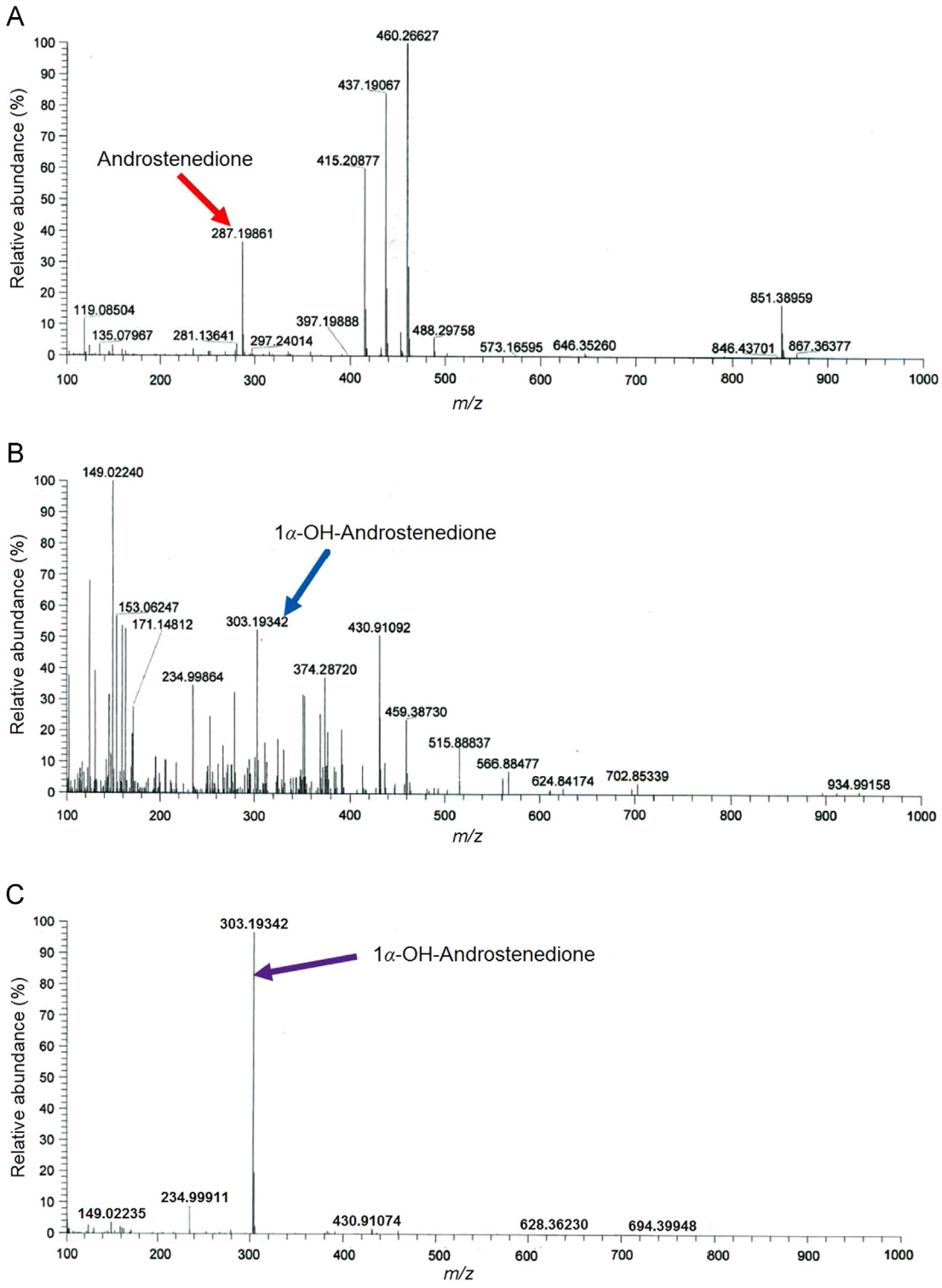


**Figure 5** ESI mass spectrums of hydroxylated product of androstenedione with the recombinant 139-3 protein (A and B) and the authentical 1$\alpha$-OH-androstenedione (C). The signals at *m/z* 287.19861(A) and 303.19342 (B and C) correspond to androstenedione (MW 286) and 1$\alpha$-OH-androstenedione (MW302).

### *3.4. Optimization of BM3 139-3 reaction conditions*

The chemical synthesis of 1$\alpha$-hydroxylated product was still a very challenging process, requiring multi-step reactions and leading to very low yield of 1$\alpha$-hydroxyandrostenedione[26]. Biocatalyst-mediated hydroxylation of androstenedione (**1**) was thus regarded as an alternative. Up to date, myxobacterial *CYP260A1*[26] and other three BM3 variants[16] were reported to have the hydroxylating capacity of androstenedione (**1**) at C-1 position. However, the yield of the 1$\alpha$-hydroxylated product catalyzed by these P450

**Table 1** NMR data for 1$\alpha$-OH-androstenedione (600 MHz for $^{1}$H NMR and 150 MHz for $^{13}$C NMR, $CDCl_3$).

| Position | 1$\alpha$-OH-Androstenedione | |
|---|---|---|
| | $\delta_C$ | $\delta_H$ |
| 1 | 75.39 | 3.99–3.93 (m, 1 H) |
| 2 | 35.66 | 2.49–2.32 (m, 2 H) |
| 3 | 198.22 | |
| 4 | 124.16 | 5.77 (s, 1 H) |
| 5 | 170.01 | |
| 6 | 32.48 | 2.49–2.32 (m, 2 H) |
| 7 | 30.98 | 0.94–0.88 (m, 1 H), 1.94–1.91 (m, 1 H) |
| 8 | 33.88 | 1.70 (m, 1 H) |
| 9 | 46.65 | 1.73 (m, 1 H) |
| 10 | 38.76 | |
| 11 | 20.03 | 1.43–1.34 (m, 1 H),1.70 (m, 1 H) |
| 12 | 31.48 | 1.02–0.95 (m, 1 H), 1.82 (1.82 (m, 1 H) |
| 13 | 45.16 | |
| 14 | 53.91 | 1.32 (m, 1 H) |
| 15 | 30.43 | 1.43–1.34 (m, 1 H), 1.95 (m, 1 H) |
| 16 | 34.23 | 2.05 (m, 1 H), 2.49 (m, 1 H) |
| 17 | 218.95 | |
| 18 | 14.8 | 0.85 (s, 3 H) |
| 19 | 17.4 | 1.15 (s, 3 H) |

enzymes was not mentioned in these articles[16,26]. Therefore, to investigate whether C-1$\alpha$ hydroxylation catalyzed by BM3 mutant *139-3* could be industrially used, an optimization of catalyzing conditions was performed. The effect of temperature, pH or incubation time on enzyme activity was thus tested (Fig. 6). As shown in Fig. 6A, BM3 mutant *139-3* displayed activity over a broad temperature range from 4 to 50 °C. With the increase of temperature below 37 °C, the hydroxylation activity of this P450 enzyme improved. When the temperature reached 37 °C, 139-3 exhibited maximum activity. Above 37 °C, the hydroxylation activity of 139-3 declined rather rapidly and only about 10% activity was retained at 50 °C (Fig. 6A).

Also, pH had an effect on the hydroxylated activity of BM3 139-3 (Fig. 6B). As illustrated in Fig. 6B, BM3 139-3 had a broad pH tolerance, ranging from 5.5 to above 10. The optimum pH of 139-3 was near neutrality. When pH was below 5.5, the hydroxylated activity of BM3 variant *139-3* was almost completely lost. With the growth of pH from 5.5 to 7, the hydroxylated activity of BM3 139-3 increased significantly. When pH reached about 7.0, the activity of BM3 variant *139-3* was the maximum. With the further increase of pH, the activity of this P450 mutant began to decline. When pH reached 10, 50% activity of BM3 mutant was retained (Fig. 6B).

The effect of incubation time on hydroxylated activity of BM3 variant *139-3* was deciphered in Fig. 6C. As shown in the time profile, with the extension of reaction time from 0 to 4 h, the activity of the enzyme increased markedly. When the reaction was performed for 4 h, the P450 BM3 reached the maximum activity. Then, as the reaction time was further prolonged, the activity of the BM3 protein began to slow down. When the crude enzyme was incubated with androstenedione (**1**) for 12 h, the enzyme remained 50% activity (Fig. 6C).

Overall, the optimal temperature, pH and incubation time of hydroxylated reaction catalyzed by P450 139-3 was optimized to be 37 °C, 7.0 and 4 h, respectively. Therefore, the hydroxylated reaction was thus performed at pH 7.0 at 37 °C for 4 h. The result indicated that 37% transformation rate was obtained.

### *3.5. Determination of the key amino acid regulating the hydroxylation activity of 139-3*

To improve the hydroxylation activity and substrates specificity of *139-3*, random mutagenesis of *139-3* was performed by EP-PCR. Four variants were thus generated (Table 2). The four variants shared a common mutated site, *R379S*. These mutants were heterologously expressed in *E. coli* for the yield of recombinant proteins, which were then used as the biocatalyst for determination of hydroxylation activity towards 13 steroids (Fig. 1). Surprisingly, these mutant *139-3* enzymes lost their hydroxylation activities towards any steroids listed in Fig. 1 completely. This evidence revealed that arginine residue located at site 379 played key role in the hydroxylation activities of 139-3. When arginine residue was mutated to serine residue, the hydroxylation activity of 139-3 towards androstenedione (**1**) was lost completely.

## 4. Conclusions

Finding new substrates with pharmaceutical potential for biocatalysts like P450 is always challenging. In this contribution, a P450

**Table 2** Representative *139-3* variants.

| Variant | Mutation | Steroids hydroxylation activity |
|---|---|---|
| *1393-1* | *R379S* | N |
| *1393-2* | *R379S/E245G* | N |
| *1393-3* | *R379S/D243G/E245R* | N |
| *1393-4* | *R379S/T246S* | N |

N: Not detected.

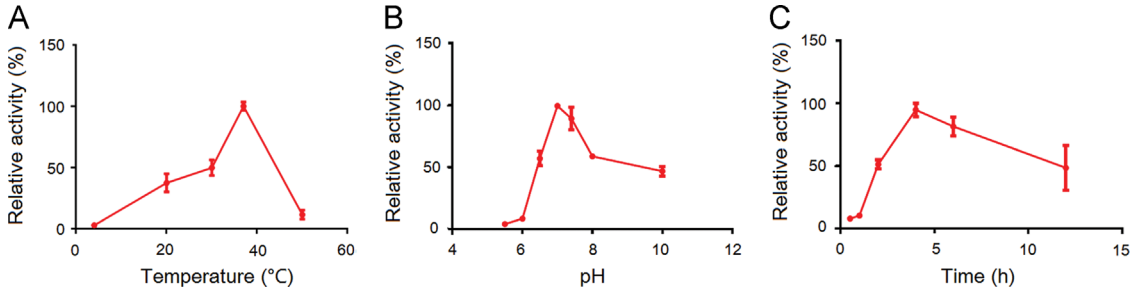


**Figure 6** Effect of temperature (A), pH (B) or incubation time (C) on enzymatic activity.

BM3 mutant 139-3 was observed to exhibit a hydroxylated activity towards androstenedione (**1**) at 1$\alpha$-position. Moreover, the maximum biotransformation rate of 37% was achieved when androstenedione (**1**) was incubated with the whole-cell biocatalyst harboring the recombinant 139-3 protein at 0.2 mol/L $Na_2HPO_4$/$NaH_2PO_4$ buffer (pH7.0) at 37 °C for 4 h. In addition, an arginine residue located at site 379 was observed to be one of the key amino acid regulating hydroxylating activity of 139-3. When argninie residue was mutated to serine residue, the hydroxylation activity of 139-3 towards androstenedione (**1**) was lost completely. Undoubtedly, these evidences widen the substrates scope of BM3, thus broadening its application potential as a biocatalyst in the biosynthesis of pharmaceutically important steroids.

## Acknowledgments

This work was supported by the Chinese Academy of Medical Sciences (CAMS) Innovation Fund for Medical Sciences (CIFMS, No. 2016-I2M-3–012), the Fundamental Research Funds for the Central Universities of Chinese Academy of Medical Sciences & Peking Union Medical College (2016ZX350001) and Beijing Natural Science Foundation (No. 7172143).

## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.apsb.2017.04.006.

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