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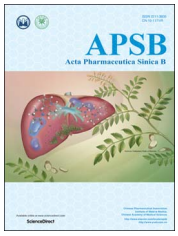

ORIGINAL ARTICLE

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

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KEY WORDS

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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 1α-position, generating a hydroxylated steroid 1α-OH-androstenedione (**1a**). To investigate whether C-1α 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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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 1α-position. Moreover, to investigate weather C-1α 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 1α-hydroxylated activity towards androstenedione (**1**) completely, thereby determining arginine residue at site 379 as one of the key amino acid regulating the 1α-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-β-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.

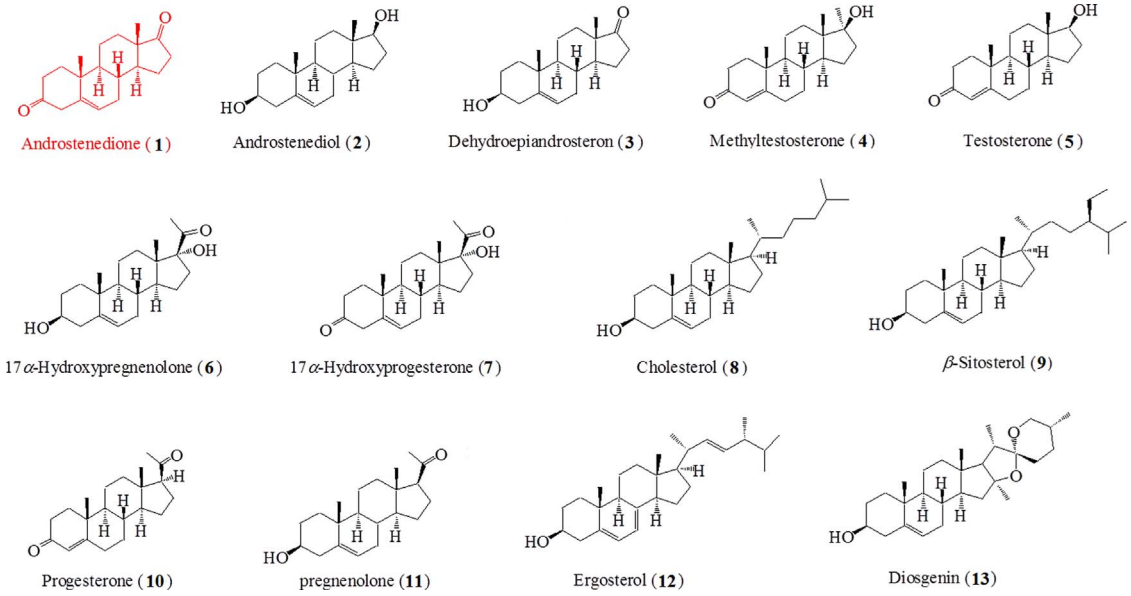

Figure 1 The structures of the steroidal substrates.

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